BIOCHEMICAL EVENTS DURING THE DEVELOPMENT OF *Pasteuria penetrans* WITHIN THE PSEUDOCOELOM OF *Meloidogyne arenaria*

By

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LIST OF ABBREVIATIONS

ELISA	Enzyme linked immunosorbent assay
BSA	Bovine serum albumin
kDa	Kilodalton
FITC	Fluorescein isothiocyanate
KLH	Keyhole Limpet Hemocyanin
M	Molar
µl	Microliter (s)
µg	Microgram (s)
mM	Millimolar (s)
µm	Micrometer (s)
mg	Milligram (s)
ml	Millileter (s)
ng	Nanogram (s)
nm	Nanometer (s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Sodium phosphate buffer
PBST	Sodium phosphate buffer plus Tween

PBST-BSA	10mM phosphate buffer pH 7.4 , 150 mM NaCl, 0.05 % Tween, 2% bovine serum albumin
SDS	Sodium dodecyl sulfate
UDC	6.0 M urea, 0.03 M dithiothreitol, 0.005 M CHES buffer pH 9.8
1.33x UDC	8.0 M urea, 0.04 M dithiothreitol, 0.00665 M CHES buffer pH 9.8
WGA	Wheat-germ agglutinin

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Chairperson: Dr. Robin M. Giblin-Davis
Major Department: Entomology and Nematology

Pasteuria penetrans is a naturally-occurring bacterial parasite of root-knot nematodes and a promising biocontrol agent. The endospores of this bacterium attach to the cuticle of second-stage juveniles and complete their life cycle within the infected female. Sequential steps required for the bacterium's propagation include: attachment, infection and germination, vegetative growth, sporulation and release. The hypothesis to be tested in these studies considers that molecular entities present on the surface of mature endospores, designated as spore adhesins, are synthesized at a certain time during the growth and sporulation of *P. penetrans*, and these allow the bacteria to attach to the nematode host. The objectives of this study were to: 1) determine the temporal relationship between adhesin epitope formation and sporulation of *P. penetrans*; 2) determine adhesin epitope distribution during spore development and association with

nematode host and; 3) determine if the adhesin epitope is shared by different species of *Pasteuria* with different host specificities. ELISA and immunoblotting showed that only proteins extracted from *P. penetrans*-infected root-knot nematodes harvested 24 days after inoculation and growth at 35 °C were recognized by the anti-P-20 IgM Mab that recognizes an adhesin epitope. Labeling, which was first observed in stage III of sporogenesis, identified the epitope distributed over the parasporal fibers, and over other structures, such as sporangium and exosporium, as the bacteria proceeded with the sporogenesis process. However, labeling was not observed on the basal rings, cortex, inner spore coat, outer spore coat, or protoplasm. Immunofluorescence revealed that the epitope does not occur uniformly on the surface of mature endospores. Immunocytochemistry and immunoblot analysis showed that the adhesin epitope is shared by other species of *Pasteuria*. The uniform distribution of the epitope over the thin sections of mature endospores of strains and species of *Pasteuria* support a role for the epitope in recognition of the nematode host as an early event in the attachment process.

CHAPTER 1
INTRODUCTION

Host: Root-knot nematodes (*Meloidogyne spp.*)

Historical Background

Berkeley (1855), in England, reported a plant disease in a greenhouse as "vibrios forming excrescences on cucumber roots". Muller (1884) described the nematode pathogen of the disease as *Heterodera radicicola.* This pathogen, root-knot nematode, was considered as a single large group for 65 years; nevertheless, it was reclassified several times during that period as follows: *Anguillula marioni* Cornu, 1879, *A. arenaria* Neal, 1889, *A. vialae* Lavergne, 1901, *H. javanica* Treub, 1885, *Tylenchulus arenarius* Cobb, 1890, *Meloidogyne exigua* Göldi, 1887, *Oxyurus incognita* Kofoid and White, 1919, *Caconema radicicola* Cobb, 1924, and *Heterodera marioni* (Cornu, 1879) Marcinowski, 1909 (Thorne, 1961). The nematodes gained much attention. There was obvious physiological and biological variability noted among different field populations (Christie, 1946, Christie and Albin, 1944). This led to the classical work by Chitwood in 1949. He re-erected the genus *Meloidogyne* Göldi, 1887 to receive all root-knot nematodes. He not only redescribed the type species, *M. exigua* (Göldi, 1887), but also redescribed *M. javanica* (Treub, 1885), *M. arenaria* (Neal, 1889), and *M. incognita* (Kofoid &White, 1919), and described *M. hapla*, and a new variety, *M. incognita* var. *acrita* (Hirschmann, 1985). A host-range study conducted by Sasser (1954) showed that

the host response to root-knot nematode infection was widely variable, not only among species, but also within species of this nematodes. This was the first report calling for the use of differential host plant bioassays to aid with the identification of *Meloidogyne* species. Taylor and Sasser (1978) modified the original list of host differentials to include the following six differential host plants: cotton (*Gossypium hirsutum* cv. Deltapine 16), peanut (*Arachis hypogea* cv. Florunner), pepper (*Capsicum annum* cv. California Wonder), strawberry (*Fragaria* × *ananassa* Duchesne), tobacco (*Nicotiana tabacum* cv. NC 95), tomato (*Lycopersicon esculentum* cv. Rutgers), and watermelon (*Citrullus lanatus* cv. Charleston Gray). Based on these differentials, four races of *M. incognita* and two races of *M. arenaria* were identified (Taylor and Sasser, 1978). In addition, two biological races in *M. hapla*, based on chromosome numbers, have been reported (Triantaphyllou, 1966). Also, some physiological (Carneiro et al., 1990) and genetic variability (Carneiro et al., 1998, Janati et al., 1982, Triantaphyllou, 1985) has been reported within *M. javanica* and *M. arenaria* (Esbenshade and Triantaphyllou, 1985). Up to 1981, about 80 species of *Meloidogyne* had been described (Eisenback et al., 1981).

Life Cycle

The life cycle of root-knot nematodes starts with the production of eggs. After the embryogenesis process is completed the first-stage juvenile molts within the egg. The second-stage juvenile (J2) hatches, and migrates freely in the soil. The J2 is the major survival stage and only infective stage. It enters susceptible plant roots to continue its life cycle. The J2 are attracted to plant roots. They migrate to a root of a susceptible plant 25

cm vertically in 10 days (Prot, 1978). The J2 generally penetrate roots directly behind the root cap; however, penetration may occur at points where lateral roots emerge (Hussey, 1985). Cellulase, derived from esophageal gland cells, may play a role in the penetration and migration in roots (Bird et al., 1975). The subventral gland cells are the most active in J2 (Bird, 1967). Nonetheless, following the onset of parasitism, the dorsal gland cell increases the production of secretory granules and becomes the predominate gland cell in females (Bird, 1968; 1983; Hussey and Mims, 1991). After penetrating a root, the J2 migrates intercellularly in the cortex toward the region of cell differentiation. When its head reaches the periphery of the vascular tissue, it establishes a feeding site (Hussey, 1985). Secretions injected through the stylet into the vascular tissue of the cells near the head cause morphological and physiological changes in these cells, which enlarge and are called giant cells (Hussey et al., 1998). The roots enlarge at those sites producing galls (Loewenberg, et al., 1960). Five to six multinucleate giant cells develop. These are highly specialized cells on which the nematode feeds (Hussey, 1985). After establishing the feeding site, the J2 becomes sedentary and undergoes morphological changes including increase in its body width but not its length (Taylor and Sasser, 1978). The nematode molts three more times during development to form the third and fourth stage juveniles and the adult stage (male and female). The males are vermiform whereas the females are globose-pyriform in shape. The rate at which these nematodes develop is influenced by several factors such as temperature, host suitability, and vigor of the host. Tyler (1933) reported that at 27.5 °C to 30 °C females developed from J2 to the egg-laying female in about 17 days, at 24.5 °C in 21 to 30 days, at 20 °C in 31 days, and at

15.4 °C in 75 days. Females reproduce mainly by parthenogenesis (Triantaphyllou, 1985). Some species are amphimitic or reproduce both by parthenogenesis or amphimixis. Females lay eggs into a gelatinous matrix that forms an egg mass. The number of eggs per egg mass is highly variable, but may range from almost 200 to 1,000 eggs. The egg masses are generally found outside the galled tissue, but in some host plants the egg mass will lie within the galled tissue.

Symptoms

Plants infected with root-knot nematodes exhibit above-ground and below-ground symptoms. The first below-ground symptoms are the formation of root galls and a poorly developed root-system. The galls result from cell enlargement (hyperplasia), and an increase in cell number (hypertrophy) surrounding the giant cells. Galls usually start to develop in 1 to 2 days after root penetration by a J2. The gall size, which can be small and discrete or large, and in some cases coalesced, is related to the number of nematodes inside the plant tissue (Dropkin, 1954). The size of the galls varies among plant species and nematode species. Generally, egg masses may be observed easily on a galled root, but in some plant species the egg masses are covered by plant tissue. Galls caused by root-knot nematodes can be diagnosed erroneously as nitrogen nodules. Nematode galls are an integral part of root tissue and can not be detached without severely damaging the roots, whereas nitrogen nodules, caused by *Bradyrhizobium* spp., are round swellings that appear to be attached to the root and are detached easily. Nodules may be infected by root-knot nematodes, and galls and egg masses can be found on the nodules (Minton and Baujard, 1990; Porter et al., 1984).

The above-ground symptoms usually depend on the initial nematode density in the soil as well as environmental conditions (Minton and Baujard, 1990). Infected plants have reduced uptake of nutrients and water, which produces yellowing, wilting, and stunting of leaves (Nestscher and Sikora, 1990).

Distribution and Economical Importance

Meloidogyne spp. are among the most widespread and important plant pathogens limiting crop productivity (Sasser and Carter, 1985). Root-knot nematodes can establish in several soil types; however, suppression of crop yields caused by these nematodes are more severe in sandy soils than in clay soils (Taylor and Sasser, 1978). Heavily infected plants may die when there is severe stress caused by hot, dry conditions. Yield losses caused by plant-parasitic nematodes are approximately $8 billion a year to producers in the United States and nearly $78 billion worldwide (Society of Nematologists, Committee on National Needs and Priorities in Nematology, 1994). However, the damage caused by root-knot nematodes alone is very difficult to determine, and sometimes it is overlooked or underestimated because of the interaction with soilborne fungi, bacteria, viruses, insects, and other nematodes (Nestscher and Sikora, 1990).

Meloidogyne spp. cause damage and are associated with many plants, including economic crops and weeds in all areas of the world (Taylor and Sasser, 1978), but they are considered to be most important in tropical regions (Johnson and Fassuliotis, 1984; Mai 1985). This is mainly due to i) high temperatures and a longer growing season that favors more generations of the nematode per year, resulting in higher nematode densities in the soil; ii) the presence of highly virulent species, such as *M. incognita, M. arenaria*,

and *M. javanica*, which are well-adapted to warmer areas, and iii) prevalence of more disease complexes involving root-knot nematodes and soilborne fungi (Mai, 1985). *Meloidogyne incognita* has the widest geographic distribution of all species described, followed closely by *M. javanica*, and *M. arenaria*. Those species are very common in tropical regions, whereas *M. hapla* is more common in temperate regions of the world (Taylor and Sasser, 1978). The optimum monthly temperature for development of *M. incognita* is 27 °C; nonetheless it can be found in areas that have an average temperature of 24-30 °C (Eisenback and Triantaphyllou, 1991). In contrast, *M. hapla* can survive in frozen soil and it can reproduce at temperatures as low as 15 °C (Taylor and Sasser, 1978).

Management

Chemical nematicides. In the 1940s, discovery of the nematicidal properties of 1,2-dichloropropane,1,3-dichloropropene (DD) made it possible to demonstrate to producers the damage caused by root-knot nematodes. It marked the beginning of the soil fumigation industry (Johnson and Feldmesser, 1987). After World War II, ethylene dibromide (EDB), 1,2-dibromo-3-chloropropane (DBCP), and bromomethane (methyl bromide, MBr) were formulated as soil fumigants. Each was offered at prices economical for use in the production of moderate to high-value crops (Johnson and Feldmesser, 1987). Later DD, EDB and DBCP were found in ground water, and were withdrawn from the market (Heald, 1987).

Since 1960, different methyl bromide formulations have been used for high-value crops. Methyl bromide has became one of the most popular fumigants because of its

broad-spectrum activity and its relatively low cost (Noling and Becker, 1994). It is not only highly efficient in the control of nematodes, but also provides control of fungi, bacteria, insects, rodents, and weeds (Thomas, 1996). Methyl bromide has been used as an agricultural soil fumigant, structural and commodity fumigant, and for quarantine and regulatory purposes (USDA, 1993a; 1993b; Watson, et al., 1992). About 79,000 tons have been used annually on a global basis by agricultural users, mainly as a soil fumigant (75%), but also as a post-harvest fumigant (22%) and for structural (3%) pest control (UNEP, 1995). Worldwide more than half of the production of methyl bromide is used on four crops: tomato, tobacco, strawberries, and melons (Ferguson and Padula, 1994; Stephens, 1996a; 1996b).

In Florida and in other states, methyl bromide is used mainly under plastic mulch as a preplant soil fumigant in the production of tomato, pepper, strawberry, other fruits, turfgrass, and nursery crops; however, most methyl bromide is consumed in the tomato, pepper, and strawberry industries (Ferguson and Padula, 1994; Johnson et al., 1962; McSorley et al., 1986; Overman and Jones, 1984).

The emission of methyl bromide into the atmosphere became a major environmental concern in the late 1980s. The Montreal Protocol Treaty, an international agreement signed by more than 150 countries, governs the world-wide production and trade of ozone-depleting substances. In 1992, the signatories of the Montreal Protocol identified methyl bromide as an ozone depleter (Watson et al., 1992). In 1993, the Montreal Protocol treaty was amended to require that developed countries freeze the production of methyl bromide at 1991 levels by 1995 (USEPA, 1993), and at the 1995

meeting, a global methyl bromide production phase-out was approved (Thomas, 1996). Industrial nations were to have a 25% reduction by 2001, a 50% reduction by 2005, and a complete phase-out in 2010, whereas developing nation should freeze the production of methyl bromide in 2002 based upon an average of the years 1995-98 (UNEP, 1995).

In the last several years, studies have been carried out to develop alternative biocides and to implement new strategies for methyl bromide replacement. Materials that have been identified to have broad spectrum activity in soils include 1,3-dichloropropene (1,3-D) products (Riegel, 2001), dazomet, trichloronitromethane (chloropicrin), dithiocarbamate (metham sodium), sodium tetrathiocarbonate, formalin or formaldehyde, and nonfumigants nematicide-insecticides (Anonymous, 1995). However, none of the materials provide the same level of broad spectrum activity as that provided by methyl bromide. Chloropicrin alone is very efficient for the control of many soilborne fungi, but it does not control plant-parasitic nematodes efficiently. 1,3-D provides control of cyst, root-knot, stubby root, lesion, ring, and dagger nematodes, but it is not effective against fungi (Locascio et al., 1997, Stephens, 1996b). 1,3-D can be mixed with chloropicrin to enhance activity against soilborne fungi. Such products are registered for more than 120 vegetable, field, and nursery crops in the United States (Melicher, 1994).

Crop rotation. Nonchemical alternatives for suppressing nematode populations include the use of crop rotation, resistant varieties, cover crops, soil amendments, flooding, solarization, bare fallowing, and biological control (Christie, 1959; Netscher and Sikora, 1990; Mai, 1985). Some of those techniques have been used for many years, and can be effective against some plant-parasitic nematodes under specific situations, but

they do not provide the same broad spectrum of control as methyl bromide.

Crop rotation is one of the oldest ways to manage *Meloidogyne* spp. However, due to their broad host range, choosing the appropriate crop can be difficult (Potter and Olthof, 1983), and in many cases the best crop choice to manage the nematode densities in the soil is not a suitable choice for the growers. The principle of this method is based on the use of resistant, susceptible, or tolerant crops for the predominant species of root-knot nematode for a specific area (Johnson, 1982). Currently, crop rotation remains an option to reduce the damage caused by root-knot nematodes in the southeastern United States (Johnson, 1982). Rodríguez-Kábana et al. (1988, 1989) showed that castor *(Ricinus communis* L.), American jointvetch (*Aeschynomene americana* L.), partridge pea (*Cassia fasiculata* Michx.), and sesame (*Sesamum indicum* L.) did not support *M. arenaria* populations in the field. McSorley et al. (1994) studied the effects of 12 summer crops on *M. arenaria* race 1 and on the yield of vegetables in microplots. Castor, cotton (*Gossypium hirsutum* L.), velvetbean (*Mucuna deeringiana* [Bort.] Merr.), crotalaria (*Crotalaria spectablis* Roth.), and hairy indigo (*Indigofera hirsuta* L.) reduced nematode numbers. Yields of vegetable crops were higher following castor than other summer crops, and yields of vegetable crops following castor as a cover crop were approximately double the yields of the same vegetable crop following peanut, a host of *M. arenaria* race 1.

Resistance. Nematode-resistant cultivars can be an option to manage root-knot nematodes, and they might be used alone or in crop rotation schemes as part of an integrated root-knot nematode control program. Attempts have been carried out to

develop cultivars resistant to one or more species of root-knot nematodes. Currently, there are nematode-resistant cultivars of tomato, southern pea, pepper, bean, and sweet potato (Noling and Becker, 1994). However, due to the occurrence of genetic variability within species of root-knot nematodes, it is difficult to develop a cultivar that is resistant to more than one race. In addition, the occurrence of mixtures of races and species of root-knot nematodes within a given area, as well as resistance being broken at high soil temperatures, often limits their usefulness. Even though the tomato resistant gene "Mi" typically confers resistance to *M. javanica*, *M. incognita*, and *M. arenaria*, virulent populations of these nematodes have completely overcome the Mi gene resistance (Castagnone-Sereno 1999; Xu et al., 2001). A greater problem to overcome is the loss of host resistance in tomato that occurs when soil temperatures heat up to over 28 °C (Abdul-Baki et al., 1996; Tzortzakakis, 1997). A loss of resistance to *M. incognita* in *Phaseolus vulgaris* was observed at 24 °C and above (Mullin et al., 1991).

Integrated pest management. The integration of different tactics have been implemented in attempts to manage plant-parasitic nematodes. In the southern United States, *M. incognita* is a major pathogen of sweet potato (Hall et al.; 1988). A combination of crop rotation, resistant cultivars, nonhost, and nematicides seems to be the most economical method of nematode control on sweet potato (Jatala and Bridge, 1990). *Meloidogyne arenaria* race 1 is one of the most serious pathogens of peanut in the southern United States. For many years peanut growers have relied on crop rotation, winter cover crops, post harvest crop destruction, and nematicides for managing root-knot nematodes (Dickson, 1998). Recently, the peanut germplasm has been released from

Texas A&M University that is resistant to race 1 of *M. arenaria* (Simpson and Starr, 1999). With the development of suitable cultivars incorporating this resistance will greatly improve nematode management for peanut producers.

Biological control agents. Root-knot nematodes, their antagonists and parasites, share the same soil habitat. Interactions of these organisms are affected by a number of factors such as the physical and chemical environment of the soil as well as the soil microflora which might play a role in the use of antagonists and parasites in root-knot nematode management (Stirling, 1991). Although several organisms such as fungi, bacteria, viruses, nematodes, mites, insects, protozoans, turbellarians, oligochaetes, and tardigrades have been shown to have some affect on nematode population densities under laboratory and greenhouse conditions, field results have been contradictory (Jairajpuri et al., 1990; Stirling, 1991). Particular attention has been given to effects of soil-inhabiting fungi on the population densities and activities of plant parasitic-nematodes. The known fungal antagonists (Gray, 1988) of nematodes are grouped as i) endoparasites of vermiform nematodes; ii) nematode-trapping fungi, and iii) female and egg parasites and cyst colonizers.

Endoparasitic fungi are classified into three categories based on their mechanism of infection and their taxonomic position: i) group I, encysting species of Chytridiomycetes and Oomycetes such as *Catenaria anguillulae*, *Lagenidium caudatum*, *Aphanomyces* sp. and *Leptolegnia* sp. which have a flagellated zoospore as their infective propagule; ii) group II, Deuteromycetes producing adhesive conidia and conidia which are ingested; and iii) group III, Basidiomycetes producing adhesive conidia. Fungi of

groups II and III initiate the infection process when the conidia either adhere to the nematode's cuticle (*Drechmeria coniospora*, *Hirsutella rhossiliensis*, *Macrobiophthora vermicola*, *Myzocytium humicola, Nematoctonus leiosporus*, *N. concurrens*, *N. haptocladus*, and *Verticillium balanoides*), or when conidia lodge in the buccal cavity or the gut of the host (all species of *Harposporium* but one) (Stirling, 1991). This latter group would not likely be efficient for biocontrol of plant-parasitic nematodes because they would be unable to ingest the conidia (Stirling, 1991).

Nematode-trapping fungi or predatory fungi have sparse mycelia that have been modified to form organs capable of capturing nematodes. They are the best known nematophagous fungi, and they have been studied for over a century (Stirling, 1991). There are six mechanisms by which these types of fungi can capture a nematode: i) Adhesive hyphae, produced by Zygomycetes (*Stylapage* and *Cystopage*) and a few species of Hyphomycetes. A yellowish adhesive secretion is produced by the fungi. These are considered to be the least sophisticated trapping mechanisms. ii) Adhesive branches produced by a few species of fungi, such as *Monacrosporium cionopagum*. Erect branches of one or two cells produced on the hyphae may anastomose to form loops or two dimensional networks, which may trap nematodes as they move around. iii) Adhesive mycelial network, the most common type of trap, found in almost all soil types. It forms from the lateral branch growing from the vegetative hypha and curving to fuse with the parent hypha. More loops are produced on this loop or on the parent hypha, until a complex, three-dimensional, adhesive-covered network of anastomosed loops is produced (*Arthrobotrys oligospora*). iv) Adhesive knobs, formed of distinct adhesive-

globose cells that are either sessile on the hypha or borne aloft on a short, erect stalk. These cells occur along the hypha, so that nematodes are often restrained by several knobs. Nematodes may struggle to escape the knobs, which may cause the knobs to detach from their stalks in some species but the knobs remain firmly attached to the nematode and germination occurs quickly. This type of trap mechanism is most common among Hyphomycetes, but it is found also in the Basidiomycetes. *Nematoctonus* produces non-detachable, hourglass-shaped knobs that are engulfed in a larger, spherical ball of viscous substance (Barron, 1997). v) Non-constricting rings, the most frequent device in nematophagous fungi. Three-celled rings are formed when erect lateral branches from vegetative hyphae thicken and curve, which then fuse to the support stalks. Nematodes are captured when rings become wedged around their bodies. vi) Constricting rings, similar to non-constricting rings. The rings are attached to hypha by short stalks. Nematodes entering these rings trigger them to swell rapidly inward, thereby capturing the nematode. The ring closes in about 0.1 second once initiated; however there is a lag period of 2 to 3 seconds from the time the nematodes first touch the ring cells until it closes. The nematodes can escape during this short period, which makes this type of mechanism an inefficient trap (Stirling 1991).

Female and egg parasites, and cyst colonizers, are a taxonomically and ecologically diverse group, ranging from host specific zoosporic fungi to opportunistic species that live largely as soil saprophytes. Over the years many fungi have been isolated from females, cysts, eggs, and egg masses of plant-parasitic nematodes, but the

majority have proved to be saprophytes rather than parasites (Chen et al., 1996; Morgan-Jones and Rodríguez-Kábana, 1988; Stirling, 1988).

Rodríguez-Kábana and Morgan-Jones (1988) listed 12 genera of fungi that are isolated frequently from females, eggs, and cysts of Heteroderidae in Australia, Europe, and North and South America: *Acremonium*, *Alternaria*, *Catenaria*, *Cylindrocarpon*, *Exophiala*, *Fusarium*, *Gliocladium*, *Nemathophora*, *Paecilomyces*, *Penicillium*, *Phoma*, and *Verticillium*. Among these *V. chlamydosporium* has been the most widely studied (Stirling, 1988), and proven pathogenic to *Meloidogyne*, *Globodera*, and *Heterodera*. The fungus *Paecilomyces lilacinus* was found parasitizing eggs of *Meloidogyne incognita* (Jatala et al., 1979) in Peru. After its discovery, it became the principal organism of interest (Dube and Smart, 1987; Jatala et al., 1979; 1980; 1981). Although it has been found in many geographical areas (Gintis et al., 1983; Godoy et al., 1983; Morgan-Jones et al., 1984; Dackman and Nordbring-Hertz, 1985) it is more common in warmer areas of the world (Domsch et al., 1980). *Paecilomyces lilacinus* has been shown to be a biocontrol agent of several species of nematodes (Jatala 1985; 1986). However, there are mixed reports on the efficacy of this fungus (Hewlett et al., 1988; Rodríguez-Kábana et al., 1984).

The bacterium, *Pasteuria penetrans* (Chen et al., 1997b; Eddaoudi and Bourijate, 1998; Freitas, 1997; Trudgill et al., 2000, Tzortzakis and Gowen, 1994; Spiegel et al., 1996), has become the most studied biocontrol agent in the last several years, and is reported to be one of the most promising biological control agents of root-knot nematodes (Chen et al., 1996; Duponnois et al., 1999; Oostendorp et al., 1991; Zaki and Maqbool,

1992). Once the problem with its cultivation and mass-production is overcome it may be a very useful biological agent in an integrated root-knot nematode management program.

Parasite: *Pasteuria penetrans*

Historical Background

The history of *Pasteuria* spp. has a rather unusual start in that the organism was first reported as a parasite of the water flea *Daphnia magna* Strauss. This discovery was made in 1887 by Elie Metchnikoff, soon after he accepted a research position offered by Louis Pasteur at the newly formed Pasteur Institute, Paris (Sayre, 1993). In 1888 Metchnikoff erected a new genus, *Pasteuria*, which he named in honor of Louis Pasteur, to contain the new species, *P. ramosa*. He emphasized the unique mode of division of this bacterium when he wrote, "*Pasteuria* sp. was able to undergo as many as five longitudinal divisions at the same time, giving it a characteristic fan shape" (Sayre, 1993 p101). All attempts made by Metchnikoff to culture the bacterium failed, and thus the type strain was not established (Sayre, 1993).

For many years the description of *Pasteuria ramosa* enticed researchers around the world to seek the bacterial parasite of water fleas (Henrici and Johnson, 1935; Hirsch , 1972; Staley, 1973). A budding bacterial species of the *Blastobacter* group, found occasionally on the exterior surfaces of *Daphnia* sp., was classified erroneously as Metchnikoff's unique bacterium, even though it did not form either endospores, mycelium or branches, was not a parasite of cladocerans, and showed a Gram-negative

reaction. This budding bacterium (strain ATCC 27377) was cultivated in vitro, and then assigned erroneously as the type species of the genus *Pasteuria* (Staley, 1973).

Eighty-nine years after Metchnikoff discovered *P. ramosa*, it was rediscovered infecting *Moina rectirostris*, a member of the family Daphnidae (Sayre, 1977). The similarity between the newly discovered bacterial strain and Metchnikoff's bacterium was very clear despite the lack of evidence of longitudinal division. Primary colonies branched and formed a cauliflower-like shape. Daughter colonies were formed by the fragmentation of mother colonies. Quartets, doublets, and single sporangia were produced from the daughter colonies. A sporangium consisted of a conical stem, swollen middle cell, and an endogenous endospore (Sayre et al., 1979; 1983).

Ten years after *Pasteuria* had been assigned erroneously as strain ATCC 27377, that strain was reclassified as *Plactomyces staleyi* Starr, Sayre, and Schmidt, 1983 (Starr et al., 1983). Starr et al. (1983) requested that the original description of *P. ramosa* Metchnikoff, 1888 be conserved and that ATCC 27377 be rejected as the type strain of *P. ramosa*. Later that request was supported by the Judicial Commission for the Code of Nomenclature of Bacteria (Judicial Commission, 1986), and further studies supported that decision (Sayre et al., 1988; 1989).

Cobb (1906) was the first to report an organism resembling *Pasteuria* sp. (numerous highly refractile spores) as a parasite of a nematode, *Dorylaimus bulbiferous*. He erroneously classified the parasite as a sporozoan. Later Micoletzky (1925) found an organism whose shape and spore size were similar to those reported in 1906 by Cobb. Micoletzky suggested that those spores belonged to the genus of a sporozoan, *Duboscqia*

Perez. Thorne (1940) described in detail an organism parasitizing *Pratylenchus pratensis* (de Man) Filipjev (later identified as *P. brachyurus* by Thorne), and on the assumption that the organism was similar to the parasite described by Micoletzky, assigned it to the genus *Duboscqia* as *D. penetrans*. However, over the years the taxonomic position of the nematode parasite has been questioned (Canning, 1973; Williams, 1960). The misplacement of the organism, now known to be a bacterial parasite of nematodes as a protozoan, persisted for almost 70 years. Mankau (1975a) reexamined the nematode parasite using electron microscopy and showed for the first time that it is a bacterium rather than a protozoan; he reassigned it to the genus *Bacillus* as *B. penetrans* (Thorne, 1940, Mankau, 1975). Nonetheless, neither flagella nor active motility were observed in *Bacillus penetrans* (Sayre and Starr, 1985). Soon more studies on the procaryotic affinities (Mankau, 1975b), biology (Mankau and Imbriani, 1975), ultrastructure (Imbriani and Mankau, 1977), and host (Mankau and Prasad, 1977) of *B. penetrans* were carried out. *B. penetrans* was never included in the "Approved Lists of Bacterial Names" (Skerman et al., 1980), thus the confusion on the classification of the bacterial nematode parasite continued.

Sayre and Wergin (1977) observed the similarity between the developmental stages of a bacterial parasite of *Meloidogyne incognita* with the original descriptions and drawings of the life cycle of *P. ramosa*. Later morphological and taxonomic reevaluations of *P. ramosa* and *B. penetrans* were provided (Sayre et al., 1983). Finally Sayre and Starr (1985) placed the bacterial parasite of nematodes in the genus *Pasteuria*,

as *P. penetrans*, due to its similarity with *Pasteuria* rather than *Bacillus*, and presented an emended description of the genus *Pasteuria* Metchnikoff.

The Genus *Pasteuria*

Species of *Pasteuria* are Gram-positive, endospore-forming bacteria. The genetic and biochemical aspects of the formation of the virulent endospores of *Pasteuria* spp. are not well understood, but the morphological aspects are (Chen et al., 1977a; Giblin-Davis et al., 1995; Sayre and Starr, 1985; Sayre 1993). These bacteria form a dichotomously branched septate mycelium. The terminal hyphae of a mycelium elongates, and then segments to form the sporangia, and eventually endospores. (Sayre and Starr, 1985). Mother colonies, which resemble a cauliflower or elongate grapes in clusters, fragment to form daughter colonies. Daughter colonies form quartets, doublets, and finally a single sporangia which enclose a single endospore (Chen et al., 1997a; Sayre and Starr, 1985). Endospores are nonmotile and resistant to desiccation and elevated temperatures (Dutky and Sayre, 1978; Stirling, 1985; Williams et al., 1989). Endospores of *P. penetrans* are cup-shaped and measure, on average 3.4 μm $\pm$ 0.2 by 2.5 μm $\pm$ 0.2 using transmission electron microscopy (Sayre 1993).

Members of *Pasteuria*

There is still considerable confusion about the taxonomy of *Pasteuria*. Over the years the criteria used to assign species to the genus have been host specificity, developmental characteristics, and size and shape of sporangia and endospores (Sayre and Starr, 1989). However, host specificity overlaps in several cases. Although sizes of

endospores and sporangia are considered to be host specific (Ciancio, 1995), endospore diameters of *P. penetrans* vary from 3.6 to 7.0 μm (Sayre and Starr, 1985).

Cross-genera hosts have been reported. For example, one isolate of *P. penetrans* reported from the United States (Mankau, 1975a; Oostendorp et al., 1990), Puerto Rico (Vargas and Acosta, 1990) and China (Pan et al., 1993) parasitizes both *Meloidogyne* and *Pratylenchus* spp. An isolate of *Pasteuria* sp. from India parasitizes *Heterodera* sp. and *M. incognita* (Bhattacharya and Swarup, 1988), whereas another strain reported from India parasitizes *Heterodera* spp., and *Rotylenchulus reniformis* (Sharma and Davies, 1996). Davies et al. (1990) reported that endospores of a *Pasteuria* sp. extracted from *H. avenae*, cereal-cyst nematode, attached to the cuticle of *H. shachtii*, *H. glycines*, *Globodera rostochiensis*, *G. pallida*, and *M. javanica*. On the other hand, *Pasteuria* sp. S-1 showed a high a level of host specificity. S-1 strain attached to *B. longicaudatus* but did not attach to any of the other nematodes, including J2 of *M. arenaria*, *M. incognita*, *M. javanica*, *H. galeatus*, and *Pratylenchus penetrans* (Giblin-Daves et al., 1995). These results were confirmed by Bekal et al. (2001). They showed that S-1 did not attached to *H. schachtii*, *Longidorus africanus*, *M. hapla*, *M. incognita*, *M. javanica*, *P. brachyurus*, *P. scribneri*, *P. neglectus*. *P. penetrans*, *P. thornei*, *P. vulnus*, or *Xiphinema* spp.

Some isolates of *Pasteuria* have been reported to attach to and develop in different life stages of the nematode host (Abrantes and Vovlas, 1988; Davies et al., 1990). Mature endospores of *P. penetrans* were observed in the peseudocoelom of J2 and males of *Meloidogyne* sp. and J2 of *H. fici* (Abrantes and Vovlas, 1988). Davies et al.

(1990) found that a *Pasteuria* sp. isolated from the cereal-cyst nematode, *H. avenae* Wollenweber, completed its life cycle in the J2 but not in females and cysts.

Different genera of nematodes have been reported to be parasitized by *Pasteuria* spp. at the same site and in the same growing season. Giblin-Davis, during a survey in South Florida, found that *B. longicaudatus*, *Meloidogyne* spp. and *Helicotylenchus microlobus* were parasitized by *Pasteuria* spp. in Collier County; *B. longicaudatus*, *Hoplolaimus galeatus*, *Tylenchorhynchus annulatus*, and *Meloidogyne* spp. in Broward County; and *H. microlobus* and *Meloidogyne* spp. in Palm Beach County.

Currently four species of *Pasteuria* have been described so far: i) *P. ramosa*, a parasite of the cladocerans (water fleas) *Daphnia pulex* Leyding and *D. magna* Strauss (Sayre et al., 1977); ii) *P. penetrans*, a parasite of root-knot nematodes (Sayre and Starr, 1985), iii) *Pasteuria thornei*, isolated from *Pratylenchus* spp. (Starr and Sayre, 1988), and iv) *Pasteuria nishizawae* (Sayre et al., 1991), a parasite of cyst nematodes (*Heterodera* and *Globodera*).

Recently, at least three new species of *Pasteuria* have been proposed, *Pasteuria* sp. designated as S-1 (Bekal et al., 2001) from *Belonolaimus longicaudatus* Rau; North American *Pasteuria* (*Heterodera glycines*-infecting *Pasteuria*) from the soybean cyst nematode, *Heterodera glycines* Ichinohe, in Urbana, IL, USA (Atibalentja et al., 2000) and one strain from the pea cyst nematode, *Heterodera goettingiana* Liebscher in Münster, Germany (Sturhan et al., 1994).

Over the years unique isolates of *Pasteuria* have been reported. For example, a large- and a small-spored isolate of *Pasteuria* spp., each from *Hoplolaimus galeatus*

(Cobb) Thorne (Giblin-Davis et al., 1990), and another isolate from *Rhabditis* sp. (Giblin-Davis pers. comm.) were collected from a bermudagrass turf in Fort. Lauderdale, Fl. Two isolates of *Pasteuria* sp.infecting different ring nematode species have been found: C-1 (Han et al., 1999), and ring nematode *Pasteuria* (Dickson, pers. comm.). A *Helicotylenchus* sp.-infecting *Pasteuria* was isolated from bermudagrass turf shipped from CA (Crow, pers. comm.). Also, three other isolates of *Pasteuria* that attach and complete their life-cycle in *Heterodera* spp. have been reported: one isolate from *H. avenae* (Davies et al., 1990); another (HCP) from *Heterodera cajani* Koshy, the pigeon pea cyst nematode (Walia et al., 1990); and another, HMP, from *Heterodera mothi*, Khan & Husain (Bajaj et al., 1997).

It is clear that there is a need to use other criteria, in addition to those already used, to determine species of *Pasteuria*. The 16S rDNA has been used to determine more precisely the taxonomic position of *Pasteuria* (Anderson et al., 1999; Atibalentja et al., 2000; Bekal, 2001; Ebert et al., 1996). Once the conditions necessary to mass produce *Pasteuria* in vitro are known, it will be possible to establish species through genetic and biochemical studies.

Systematics and Phylogeny of *Pasteuria*

In 1992 13 genera of endospore-forming bacteria were known (Table 1.1). The basis for separating them was morphology, physiology, and genetic diversity (Berkelwy and Ali, 1994). Currently, bacteria are differentiated based on the generally accepted rule that bacteria with DNA base compositions differing by more than 10 mol %GC (G+C) should not be considered as members of the same genus. Strains differing by more than

5%GC values should not be regarded as the same species (Bull et al., 1992). The genera *Bacillus*, *Clostridium*, and *Desulfotomaculum* are very heterogenous (Table 1.1). The genera *Oscillospira* and *Pasteuria* (four species) have not yet been grown successfully in pure culture. The description of *Oscillospira* species, *O. guillermondii* (Berkely and Ali, 1994), was based on morphological characters, whereas the species of *Pasteuria* were described based on morphological characters, morphometrics, ultrastructure, and host specificity. Otherwise their DNA base composition are unknown.

In the summer of 1992 and throughout 1993 and 1994, *P. ramosa* was re-discovered parasitizing *D. magna* collected from several ponds in London, UK (Stirnadel and Ebert, 1997). Ebert et al. (1995) used these spores of *P. ramosa* collected from *D. magna*, *D. pulex*, and *D. longispina* in the previous three summers from England as well as Russia to establish the culture of *P. ramosa* by co-cultivation in *D. magna*. These authors ended the uncertainty about the phylogenetic position of *Pasteuria* Metchnikoff by sequencing the 16S rDNA of the bacterium. They provided strong evidence that *P. ramosa* belongs to the low G+C Gram-positive endospore-forming bacteria and resides within a clade containing *B. tusciae*, *Alicyclobacillus cycloheptanicus*, and *A. acidocaldarius*, as the closest neighbors. They rejected the placement of *P. ramosa* in the Actinomycetales. Anderson et al., (1999) provided the first 16S rDNA gene sequence analysis of *P. penetrans*, and showed that it is correctly placed in the genus *Pasteuria*. The authors found that *P. ramosa* is the closest neighbor of *P. penetrans*, and it is within a clade that includes *A. acidocaldarius, A. cycloheptanicus*, *Sulfobacillus* sp., *B. tusciae*,

Table.1.1. Described genera of endospore-forming bacteria and their DNA base composition.

Genus	Mol% GC
Alicyclobacillus	52-60
Amphibacillus	36-38
Bacillus	32-69
Clostridium	22-54
Desulfotomaculum	38-52
Oscillospira	—
Pasteuria	—
Sporohalobacter	30-32
Sporolactobacillus	38-40
Sporosarcina	40-42
Sulfobacillus	54
Syntrophspora	38
Thermoactinomyces	52-55

Source: Berkeley and Ali, 1994.

B. schlegelii, and *P. ramosa*. Also Atibalentja et al. (2000), using a sequence of the 16S rDNA, showed that a *Heterodera.glycines*-infecting *Pasteuria* (*Pasteuria* sp. NA) and *P. ramosa* form a distinct line of descent within the *Alicyclobacillus* group of the Bacillaceae.

Distribution

Pasteuria spp. have been reported in 51 countries and in various islands in the Atlantic, Pacific, and Indian oceans associated with 205 nematodes species belonging to 96 genera (Sayre and Starr, 1988; Sturhan, 1985). An updated host record list is reported by Chen and Dickson (1998).

Biological Control Potential

There are certain attributes that make *P. penetrans* a desirable biological control agent: 1) endospores are resistant to desiccation, high temperature, and most nematicides (Dutky and Sayre, Freitas 1997; 1978; Stirling, 1985; Williams et al., 1989); 2) encumbered nematode juveniles have reduced activity and ability to infect roots (Sturhan, 1985); and 3) infected juveniles complete their life cycle, but females have low or no fecundity (Bird, 1986; Bird and Brisbane, 1988).

Pasteuria penetrans has been shown to control root-knot nematodes in greenhouse tests (Brown and Smart, 1985; De Leij et al., 1992; Stirling 1984) and in field microplots (Brown et al., 1985, Chen et al., 1997b; Dube and Smart, 1987; Oostendorp et al., 1991; Stirling, 1984; Tzortzakakis and Gowen, 1994; Trudgill et al., 2000). Suppression of root-knot nematodes by *P. penetrans* has been observed in vineyards more than 10 years old in Australia (Stirling and White, 1982) as well as India (Mani et al.,

1999), and also in peanut and tobacco fields infected by root-knot nematodes in Florida (Dickson, pers. comm.). Also, suppression of *B. longicaudatus* by *Pasteuria* sp. S-1 in a bermudagrass turf field in Florida has been reported (Giblin-Davis et al., 1995; 2000).

Studies have been carried out to determine the optimum endospore densities to suppress root-knot nematodes (Chen et al., 1996; Melki et al., 1998; Oostendorp et al., 1991). Chen et al. (1996) found that 10,000 endospores/g of soil was necessary for suppression of *M. arenaria* race 1 on peanut in plots on a fine sand soil. Melki et al.(1998) reported that the cultivation of a susceptible host for more than one season was needed for *P. penetrans* to build up its densities to suppressive levels. Oostendorp et al., 1991 showed that endospore attachment to *M. arenaria* race 1 increased from 0.11 to 8.6 spores/J2 in plots over a 2-year cropping sequence with peanut (summer) and rye, vetch or fallow (winter)

The use of air-dried soils infested with *P. penetrans* was one of the first attempts to show the biological control potential of this bacterium. Mankau (1973) used air-dried soil infested with the bacterial spores in greenhouse studies. He reported that after 70 days, plants in the endospore-infested soil had more leaves, greater dry weight, and lower numbers of root galls than in those soil-free of endospores. However, the use of infested soil as a source of endospores is time consuming and inconvenient to transport and handle. Stirling and Wachtel (1980) reported for the first time the use of infested root powder as a source of endospores and as a method for their mass production. The authors showed that when they used 100 mg/kg of soil of air-dried and finely ground roots

containing 2×10^9 spores/g, that within 24 hours, 99% of the J2 of *M. javanica* in the pot had endospores attached to their cuticles. Stirling (1984) used tomato roots containing *P. penetrans*-infected females of *M. javanica* to produce infested, air-dried root powder. Significant control was obtained when at least 80% of the bioassayed J2 were encumbered with 10 or more spores per J2. When the infested root powder was incorporated into root-knot nematode-infested field soil at the rate of 212-600 mg per kilogram of soil, the number of galls and nematodes in the soil at harvest was significantly reduced. Also, the application of *P. penetrans* in air-dried powdered roots at 55 000 spores/cm^3 soil in pots infested with 420 J2 significantly suppressed root galling and egg production of *M. javanica* through two successive tomato growing seasons. At planting, there was an average of 14 spores per J2 in the soil (Gowen et al., 1998). The application of air-dried root powder infested with *P. penetrans* strains P-20 and P-100 has been used at Disney World at The Land, Lake Buena Vista, Florida to effectively control *M. arenaria*, and *M. incognita* over the several years on sandy plots (Dickson, pers. comm. and Brito, pers. observation).

The Effect of Other Microorganisms and Pesticides on *Pasteuira*

Duponnois and Ba (1998) studied the influence of soil microflora on the antagonistic relationship between *P. penetrans* and *M. javanica*. The authors showed that the attachment of *P. penetrans* to J2 of *M. javanica* was higher in the presence of larger soil microbial populations, such as fluorescent strains of *Pseudomonas*, nematophagous and mycorrhizal fungi. One of the explanations given by those authors was that those soil microorganisms may change the soil ionic environment, which favored the attachment of

endospores to the nematode cuticle, which is negatively charged (Himmelhoch et al,. 1979). Duponnois et al. (1999) studied the interaction of *Enterobacter cloacae* and *Pseudomonas mendocina*, which had been isolated previously from the rhizosphere of tomato cv Roman growing in a field infested by both *M. javanica* and *P. penetrans* . Those authors found that *P. mendocina* and *E. cloacae* stimulated plant growth, inhibited the reproduction of *M. incognita*, and increased the attachment of *P. penetrans* in vitro. *Enterobacter cloacae* increased significantly the reproduction of *P. penetrans*. They suggested that the introduction of *E. cloacae* in soils could enhance the efficacy of *P. penetrans*.

The compatibility of *P. penetrans* with some pesticides increased its potential to be used in an integrated management of root-knot nematodes (Brown and Nordmeyer, 1985; Freitas, 1977; Singh and Dhawan, 1998). Carbofuran had no effect on the reproduction of *P. penetrans* (Brown and Nordmeyer, 1985; Singh and Dhawan, 1998). Freitas (1977) found that treatment with 1,3-dichloropropene (1,3-D) + 17% chloropicrin, 1,3- D + 25% chloropicrin and 1,3-D + 35% chloropicrin reduced significantly the percentage of female nematodes with *P. penetrans*, whereas metham sodium did not have any effect. However, the author reported that the percentage of nematode females infected by *P. penetrans* was significantly lower (1.67%) in the soil treated with methyl bromide + 33% chloropicrin than in the untreated control (27.50%) under greenhouse conditions. Under field conditions, the percentage of females infected with *P. penetrans* from a plot treated with methyl bromide + 33% chloropicrin was 5% compared to the untreated control plot, which had 58% of the females infected (Freitas 1997). The

exposure of endospores to the fungicides, hymexazol, fosetyl-Al, and carbendazin had no effect on the attachment or development of endospores (Melki et al., 1998).

Life Cycle

Attachment of endospores to the nematode host. Endospores of the *P. penetrans* attach to second-stage juveniles (J2) of root-knot nematodes as they move through soil pore spaces. After attachment, the sporangial wall and exosporium of the majority of endospores slough off (Sayre and Starr, 1985). The bacterium is reported to attach to J2 and produce virulent endospores only within the pseudocoelom of a mature female. However, one isolate of *P. penetrans* attached to and developed within the pseudocoelom of juveniles, males, and females of *M. acronea* isolated originally from cotton (Page and Bridge, 1985). Also, an endospore-filled J2 of *Meloidogyne* sp. was isolated from a suppressive soil infested with *P. penetrans* in Florida (Dickson, pers. comm.). Stirling et al. (1990) showed that the number of endospores attached to the cuticle of J2 increased in proportion to both endospore-concentration and time. Davies et al. (1988) found that the number of J2 entering the plant host root was reduced when they were encumbered with 15 or more spores . Ahmed and Gowen (1991) reported that 11 or more endospores per J2 reduced the capability of *M. incognita*, *M. javanica*, and *M. graminicola* to enter the host roots.

Attachment is one of the major steps toward successful development of *P. penetrans* within its host, and it has been studied in several laboratories (Afolabi et al., 1995; Bird 1989; Charnecki, 1997, Davies et al., 1996). Persidis et al. (1991) used polyclonal antibodies selected against whole endopsores and wheat germ agglutinin as a

probe, and suggested that proteins glycosylated with N-acetylglucosamine are involved in the attachment. Similar results were obtained using a monoclonal antibody raised to whole endospores of P-20 isolate of *P. penetrans* and wheat germ agglutinin (Charnecki, 1997, Charnecki et al., 1998). Mohan et al. (2001) found that fibronectin-like residues on the cuticle of *M. javanica* is involved in the attachment of endospores. Other forces such as hydrophobic interactions may be involved in the attachment of endospores to its host (Afolabi et al., 1995; Davies et al., 1996; Esnard et al., 1997)

Germination. Unknown factors trigger the germination of the endospore and the formation of a germ tube. A germ tube emerges through a central opening in the basal attachment layer after an endospore-encumbered juvenile enters a host root and begins feeding (Sayre and Wergin, 1997; Sayre and Starr, 1985; 1988; Serracin et al., 1997). The germ tube penetrates the nematode cuticle and hypodermal tissue, and then enters the pseudocoelom (Sayre, 1993) where unknown growth factors promote its development into a vegetative, spherical colony, containing a dichotomously branched and septate mycelium (Satrr and Sayre 1988). The peripheral fibers of the endospores are closely associated with the cuticle of the nematode (Sayre and Starr, 1985) and are involved in the attachment of endospores to the cuticle.

Vegetative stage. When intercalary cells in the microcolony disperse, many daughter colonies are formed. Eventually quartets of developing sporangia predominate the pseudocoelom, and then the quartets separate into doublets of sporangia, which separate into single sporangia that will eventually form the endospores (Sayre 1993).

Endospore formation. The formation of bacterial endospores is a regulated and complex process. The initiation of sporulation is triggered by several genes, *spoO* genes, in response to nutrient deprivation (Foster, 1994). It is hypothesized that molecular functions that control sporulation are the same across all genera of endopsore-forming bacteria. Small acid-soluble proteins (SASPs) have been shown to be synthesized by spores of species of *Bacillus*, *Clostridium*, and *Thermoactynomycetes* during sporulation (Setlow, 1988; Setlow and Waites, 1976). The main types of SASPs found in *B. subtilis* are termed the α/β type (Connors et al., 1986) and γ type (Hackett and Setlow, 1984), which are synthesized during the first 3-4 hours of sporulation, and are found only in spores (Setlow et al., 1992). Previous studies indicated that α/β type-SASPs are DNA-binding proteins, and their binding to the DNA cause UV resistance by modifying spore DNA's UV photochemistry (Manson and Setlow, 1986; Setlow and Setlow, 1987). Another molecule that is found in spores but not in vegetative cells is the dipicolinic acid, which is located in the core of the endospores (Madigan et al, 1997). Studies have shown that calcium, which is present in high concentration in spores, forms a complex with dipicolonic acid in the core, and confers the heat resistance found in endospores (Madigan et al., 1997).

The factors that trigger the sporulation of *P. penetrans* within the pseudocoelom of the nematode host are not known. However, the sequence of morphological events during the endogenous spore formation of *P. penetrans* is similar to other Gram-positive endospore-forming bacteria (Chen et al., 1997a; Sayre 1993) as follows: i) formation of a transverse septum within the endospore mother cell; ii) condensation of a forespore from

the anterior protoplast; iii) formation of a multilayered wall about the forespore; iv) lysis of the old sporangial wall; and v) release of an endospore (Sayre 1993).

Chen et al. (1997a) found that the sporogenesis process of *P. penetrans* generally matched stages II through VII following vegetative growth reported for *Bacillus thuringiensis*. Stage I is unique for *Pasteuria* sp. The stages are as follows: 1) stage I, mycelium dichotomously branched and microcolonies fully septate; terminal cells elongate to form a sporogenous cell; 2) stage II, the terminal cells increase in size and become oval, 1.2 to 1.7 μm by 0.6 to 1.0 μm, bounded by a 0.002 μm-thick wall; a membrane is formed about 0.4 μm from the anterior end and separates the forespore from the parasporium; 3) stage III, parasporium increases in size and engulfs the forespore. Parasporal fibers are formed and attach to the lower part of the forespore. An inner membrane defines the forespore protoplast and an outer membrane defines the mother cell's protoplast; 4) stage IV, lamella, which rises from the cortex, and inner and outer spore coats start to form; 5) stage V cortex with formation of inner and outer spore coats; the inner spore coat is a narrow multilaminar band whereas the outer spore coat is a wide electron-dense wall; 6) stage VI, formation of exosporium, a delicate membrane that delimits the outermost layer of a typical Gram-positive bacterium; 7) stage VII, complete maturation with formation of endospore, the basal ring surrounding the germinal pore. An epicortical layer, which is a discontinuous, electron-dense band was observed between the cortex and the inner spore coat. Endospores of *P. penetrans* measure an average of 3.4 μm ± 0.2 by 2.5 μm ± 0.2 (Sayre 1993).

The life cycle of this bacterium is not completely synchronized with the life cycle of the nematode since it is possible to observe different developmental stages simultaneously at a given time within the pseudocoelom of a single root-knot nematode female (Chen et al., 1997a). The rate of development is highly temperature-dependent (Hatz and Dickson, 1992; Serracin et al., 1997; Stirling 1981). The optimum temperature for the development of the *P. penetrans* was 35 °C, at which the bacterium completed its life cycle in 35 days after inoculation (Hatz and Dickson, 1992). An average of 2×10^6 endospores have been found within one single female of *P. penetrans*-infected *Meloidogyne* sp. (Sturhan, 1985) and *P. penetrans*-infected *M. javanica* (Stirling 1991).

Soil phase. Endospores are released into soil upon host disintegration. Endospores are not actively motile in soil; therefore, its contact with the nematode host must rely on the motility of J2, as well as physical factors affecting endospore distribution (Sayre 1993). The factors that mediate the movement and survival of endospores of *P. penetrans* in soil are not well understood. However, soil water percolation, sizes of soil pore openings, surface charge of soil particles, tillage practices, and soil microflora may play important roles in the distribution of endospores (Sayre, 1993). Kamra and Dhawan (1998) found that at pH 8.0 to 10.0, the average number of endospores encumbered on the bioassayed J2 of *H. cajani* was 36 and 26 compared to 10 and 7.0 at pH 6.0 and pH 4.0, respectively. Those authors also showed that the movement and distribution of endospores in soil increased with greater pore size, and decreased with an increase in the silt and clay contents of the soil.

Host Specificity

Host specificity has been used for many years to determine species of *Pasteuria*. According to Sayre and Starr (1985; 1989) host range of *P. penetrans* is limited to species of *Meloidogyne*.

Host specificity of *P. penetrans* has been reported in most cases by observing only the attachment of endopores to its host rather than by establishing infection and production of mature endospores. Attachment can occur, but endospores might fail to germinate and propagate within the nematode. Duponnois et al. (2000) tested 25 isolates of *P. penetrans*, and found that only six attached, developed, and produced mature endospores in *M. incognita*.

Attachment of endospores was greater to the nematode species from which endospores were originally cultured (Oostendorp et al., 1990; Somasekhar and Metha, 2000). When labeling endospores using monoclonal antibodies, larger areas of the endospores were labeled when *Pasteuria* were reared on the same nematode population from which endospores were taken than from other populations of root-knot nematodes (Davies et al., 1994). However, Davies et al. (1988) found that a particular isolate of *Pasteuria* sp. adapted and shifted from one nematode host to another by continually culturing the bacterium on a given nematode host. Stirling (1985) reported that attachment was not always related to the species from which the endospores were isolated, or to the species of the recipient nematode.

Davies et al. (2001) using only the PP1 strain of *P. penetrans* and several field populations of root-knot nematodes collected from Burkino Faso, Ecuador, Greece,

Malawi, Senegal, Trinidad, and Tobago showed that the extent of attachment differed between countries. Also, those authors found similar results when endospores of *P. penetrans* collected from those countries were assayed against *M. arenaria* and *M. incognita*.

Endospores of *P. penetrans* did not attach to the entomopathogenic nematodes *Steinernema glaseri* Steiner, *Heterorhaditis zealandica* Poinar and *H. bacteriophora* Poinar and seven new isolates each of *Steinernema* sp. and *Heterorhaditis* sp. when juveniles were exposed to 1×10^5 spores/ml for 24, 48, and 72 hours at 25 °C (Somasekhar and Metha, 2000). Similar results were observed by Mendoza de Gives et al. (1999), who reported that *P. penetrans* did not attach to animal-parasitic nematodes, free-living nematodes, including wild type *Caenorhabditis elegans* (Maupas) Dougherty and three of its surface (*srf*) mutants. Oostendorp (1990) also showed that endospores of *P. penetrans* did not attach to the free-living nematodes, *Panagrelus redivivus* (L.) Goodey and *C. elegans*, but attached to different species of plant-parasitic nematodes.

The nature and the amount of protein on the surface of endopores may explain host specificity (Davies et al;. 1992; Persidis et al., 1991). Monoclonal antibodies have shown that the surface of endospores of *P. penetrans* isolated from *M. incognita* race 1 is highly heterogenous (Davies et al., 1994). These and other studies (Davies and Redden, 1997) have suggested that the virulence of the bacterium to a certain species of root-knot nematodes is dictated by the surface properties of endospores, and suggested that similar heterogeneity will be present in the nematode cuticle. Differences in cuticle characteristics of J2 of root-knot nematodes have been reported (Davies and Danks

(1992). Charnecki et al., 1998 showed that the anti-P-20 IgM MAb recognized differences in the protein extracts from B4, P-20, and P120 isolates of *P. penetrans*, which have different host specificities.

Cultivation

Pasteuria *spp. have not been grown successfully in pure culture (Reise et al.,* 1988; Williams et al. 1989; Bishop and Eller, 1991). Currently *P. penetrans* produces virulent endospores only within the pseudocoelom of females of *Meloidogyne* spp., which in turn must be reared on the roots of a plant host or on excised-root systems (Verdejo and Jaffee, 1988). The mass production of this bacterium relies, currently in the use of dried, powdered roots obtained from infected root systems grown in a greenhouse (Stirling and Wachtel, 1980).

Interaction: Host-Parasite

The Role of Adhesin Proteins in the Host-Parasite Relationship

The surface of Gram-positive bacteria has adhesin proteins, also known as virulence factors, that allow the bacteria to adhere, invade, and colonize tissues (Salyers and Whitt, 1994). Studies on the composition of the surface proteins have focused mainly on pathogenic bacteria (Kehoe, 1994).

Virulence factors are classified into two major categories: i) promoters of bacterial colonization and invasion of the host; and ii) those that cause disease in the host. Among the virulence factors that promote bacterial colonization are pili, or fimbriae (Robins-Browne, 1994; Salyers and Whitt, 1994; Suoniemi et al., 1995), and afimbrial adhesins

(Salyers and Whitt, 1994) that adhere to mucosal surfaces and bind tightly to the host cells, respectively. *Streptococcus pyogenes* has a nonfibrillar adhesin (protein F) that mediates its attachment to fibronectin, a protein found on many host cell surfaces, including the mucosa of the human throat (Salyers and Whitt, 1994).

Several environmental signals may affect virulence. These may include temperature, carbon source, osmolarity, starvation, stress, pH, growth phase; and the levels of specific nutrients including iron, calcium, sulfate, nicotinic acid, and specific amino acids (Mekalanos, 1992). Bacteria use different sigma factors to control different set of genes under specific conditions (Salyers and Whitt, 1994). Similar mechanisms might be used by *P. penetrans* to produce endospore adhesins involved in recognition and attachment to the nematode host.

Objectives

The biochemical events that occur during the development of *P. penetrans* within the root-knot nematodes' pseudocoelom are poorly understood and may provide valuable insight into the conditions necessary for the formation of virulent endospores.

The objectives of this research project were to 1) determine the sequence of events required for the formation of *P. penetrans* spore-associated proteins (adhesins) that are required for the attachment of endospores, as a function of the development of *P. penetrans* within its nematode host, *M. arenaria* race 1; 2) determine the distribution of an adhesin-related epitope on the surface of virulent endospores; 3) detect and localize antigens bearing the epitope during the sporogenesis process; and 4) determine whether or

not different species or isolates of *Pasteuria* share the same adhesin-related epitope, which is recognized by the anti-P20 IgM MAb. In addition, a polyclonal antibody against a synthetic polypeptide, which was designed according to the conserved regions of small, acid-soluble proteins (SASPs) of *Bacillus* spp. was prepared for use as a probe to detect SASPs as a development marker in the sporulation process in *P. penetrans*.

CHAPTER 2
SYNTHESIS AND IMMUNOLOCALIZATION OF AN ADHESIN-ASSOCIATED EPITOPE IN *Pasteuria penetrans*

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr is a Gram-positive, endospore-forming bacterial parasite of *Meloidogyne* spp. Endospores attach to second-stage juveniles (J2) as they move through soil pore spaces. Unknown factors trigger infection of the nematode host and germination of the endospore. The germination of the endospore occurs after the endospore-encumbered juvenile enters host roots and begins feeding (Sayre and Wergin, 1997; Sayre and Starr, 1985, 1988; Serracin et al., 1997) at some point in development, presumably before the J2 molts to the third-stage juvenile. A germ tube penetrates the nematode cuticle and hypodermal tissue, and then enters the pseudocoelom (Sayre and Starr, 1988), where unknown growth factors promote vegetative growth, differentiation, sporulation, and maturation of endospores. Endospores are released into soil upon host disintegration, and more than 2 million endospores have been found within one single *P. penetrans*-infected *Meloidogyne* sp. female (Sturhan, 1985).

There are certain attributes that make *P. penetrans* a desirable biological control agent: 1) endospores are resistant to desiccation, high temperature, and most nematicides (Dutky and Sayre, 1978; Stirling, 1985; Williams et al., 1989); 2) encumbered juveniles

have reduced activity and ability to infect roots (Sturhan, 1985); and 3) infected juveniles complete their life cycle, but females have low or no fecundity (Bird, 1986; Bird and Brisbane, 1988). These bacteria complete their life cycle and produce virulent endospores only within the pseudocoelom of *Meloidogyne* spp., which in turn must be reared on a plant host either in pots or on excised-root systems (Verdejo and Jaffee, 1988). Attempts to culture *P. penetrans* in vitro have failed to produce virulent endospores (Reise et al., 1988; William et al., 1989; Bishop and Ellar, 1991). The biochemical events that occur during the development of *P. penetrans*, leading to the formation of virulent endospores within the pseudocoelom, are poorly understood.

The molecular basis for the recognition and attachment has been the subject of investigation in several laboratories. Lectin-carbohydrate interactions have been suggested to be involved in the attachment of *P. penetrans* to its nematode host. Previous studies have shown that wheat-germ agglutinin (WGA) inhibited the attachment of endospores (Bird et al., 1989; Charnecki 1997; Charnecki et al., 1998; Davies and Danks, 1993). Also, proteins extracted from endospores of *P. penetrans* were recognized, not only by monoclonal antibodies (Charnecki 1997; Charnecki et al., 1998; Davies and Redden, 1997) and polyclonal antibodies selected against whole endospores of *P. penetrans* (Charnecki et al., 1998; Chen, S. Y et al., 1997; Davies et al., 1992; Persidis et al., 1991), but also by wheat-germ agglutinin (WGA) (Bird et al., 1989; Charnecki, 1997; Persidis et al., 1991). These results indicate that one or more epitopes detected by the antibodies may be glycosylated with β-1-4 linked-acetylglucosamine.

Understanding the processes that lead to the growth, differentiation, sporulation, and maturation of *P. penetrans* within the pseudocoelom will likely provide a basis to establish the conditions required for its mass production in vitro. The objectives of this study were to (1) determine the synthesis of spore-associated proteins (adhesins) as a function of *P. penetrans* development within the pseudocoelom of the nematode host, *M. arenaria* race 1; (2) determine the distribution of an adhesin-associated epitope on the surface of virulent endospores; and (3) detect and localize an adhesin-associated epitope during the sporogenesis process.

Materials and Methods

Nematode Source

Meloidogyne arenaria (Neal) Chitwood race 1 used in this experiment was isolated originally from peanut (*Arachis hypogea L.*), Green Acres Research Farm, University of Florida, Alachua County, Florida. The nematode was reared on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) maintained in a greenhouse. Eggs of the nematodes were extracted from galled roots by dissolving the gelatinous matrix with 0.5% NaOCl for 20 seconds and collecting the eggs on a sieve with 75 µm-pore openings (200 mesh) nested in a sieve with 25-µm-pore openings (500 mesh) (Hussey and Barker, 1973). Second-stage juveniles were obtained by hatching the eggs in a modified Baermann funnel (Pitcher and Flegg, 1968). Juveniles (up to 3-day-old) were collected on an autoclaved 500-mesh sieve.

Pasteuria penetrans Source

Pasteuria penetrans strain P-20 (Oostendorp et al., 1990) used in this study was collected originally from females of *M. arenaria* race 1 parasitizing peanut in Levy County, FL and reared on *M. arenaria* race 1 growing on tomato in a greenhouse. One to three-day-old juveniles (J2), with endospores attached to their cuticles were obtained by incubating them with a suspension containing 1×10^5 endospores/ml overnight, with constant aeration at room temperature. Endospores were exposed to a mild sonification (FS14, Fisher Scientific, Suwanee, GA) for 5 minutes before attachment. Twenty spore-encumbered J2 were chosen randomly from a glass-slide mount, and the number of endospores attached per J2 was estimated with an inverted compound microscope at 400×. The percentage of endospores attached was 100% with an average of 7 ± 3 endospores per juvenile. Tomato plants (45-day-old seedlings) growing in 15-cm-diam. clay pots, were inoculated with endospore-attached J2 (3,000 J2/plant). Three days later, the plants were inoculated again as before. Plants were fertilized twice a week by watering them with a solution containing 0.63 g of 20-20-20 (N-P-K) (Peters Professional, general purpose fertilizer, Division, United Industries Corp., St. Louis, MO) per liter. Water and insecticide applications were provided as needed. At 45 to 60 days after inoculation, the root systems were harvested, washed with tap water and weighed. Roots were cut into pieces 2 to 5 cm long and subjected to digestion in a 1-liter Erlenmeyer containing Rapidase Pomaliq 2F at 1:5 (g/v) (Gist Brocades Pomaliq product number 7003-A/DSM Food Specialities USA Inc., Menominee, WI), previously optimized with a buffer system (Charnecki, 1997), and agitated on a shaker at 120

oscillations per minute for approximately 24 hours at room temperature. Softened roots were placed in a sieve with 600 µm-pore openings (30 mesh) nested in a sieve with 150-µm-pore openings (100 mesh) and sprayed with a heavy stream of tap water according to Hussey (1971), with modifications. Females and root debris were collected in a beaker by washing the sieve with a jet of deionized H_20, and the contents centrifuged through 20% sucrose (w/v) at 1,500 × *g* for 5 minutes; the pellet fraction was centrifuged again through 47% sucrose (w/v) (Chen et al., 2000). The supernatant containing the females was collected in a beaker and the females were examined for *P. penetrans* infection with an inverted microscope at 100×. Endospore-filled females were hand-picked with forceps under a dissecting microscope at 40× (Nikon, Marietta, GA), and placed in a 1.5 ml siliconized microtube containing 300 µl of deionized H_20. Infected females were washed three times in deionized water by centrifugation at 10,000 × *g* for 2 minutes. Endospores were collected by grinding the females with a sterile pestle, and the suspension filtered through a nylon filter either with 21 µm or 18 µm openings (Spectra/Mesh). The concentration of endospores was determined by counting three 10 µl aliquots using a hemocytometer (Fisher Scientific) at a magnification of 450×. Endospores retained on a sieve with 21 µm openings were stored at 4 °C, and used as inoculum for further production of the bacterium, whereas the endospores retained on a sieve with 18 µm openings were stored at - 20 °C and used for protein extraction.

Experimental Design

Two sets of J2 of *M. arenaria*, one exposed and the other unexposed to *P. penetrans* endospores, were compared with respect to development. These were arranged

randomly, with four replications per treatment per each designated "window of *P. penetrans* development" (harvest time: 12, 16, 24, and 38 days after inoculation). The windows of development were based on those reported by Hatz and Dickson (1992) and Serracin et al. (1997). 'Rutgers' tomato seedlings growing in a clay pot (10-cm-diam.) containing autoclaved sand were inoculated with 3,500 J2/plant (≤ 2 days old) with and without endospores attached. Plants were maintained in a growth chamber at 25 °C for 48 hours to allow the nematodes to enter roots. After 48 hours the plants were removed from pots, and the roots washed thoroughly with tap water to remove any juveniles that had not penetrated. The seedlings were replanted in clay pots (15-cm-diam.), placed in a growth chamber at 35 °C, and exposed to a 12-hour-day photoperiod. Plants were harvested at 12, 16, 24, and 38 days after inoculation. The root systems harvested from plants were washed in tap water, dried with a paper towel, weighed, cut into pieces 2 to 5 cm long, and incubated in an aqueous solution of commercial Rapidase Pomaliq 2F (Charnecki, 1997). Nematodes and softened roots were collected on a sieve with 600-µm-pore openings (30 mesh) nested in a sieve with 25-µm-pore openings (500 mesh), and washed as before. The nematodes were transferred to a sterile beaker, and twenty nematodes were hand-picked from each root system. To determine the percentage of nematodes infected by *P. penetrans*, and the stage of development of the bacterium from those nematodes, these were crushed individually in a 2.5 µl drop of lactophenol and 1% methyl blue (w/v) (Sigma, St. Louis, MO) (Serracin et al., 1997) under a cover glass on a glass slide, and examined with an inverted microscope (Nikon) at 400× magnification. The remaining uninfected and infected nematodes from each harvest time were hand-

picked, washed, and stored in 1.5 ml siliconized microtubes containing 10 µl PBS (10 mM sodium phosphate buffer, 0.15 M sodium phosphate), pH 7.2 at −20 °C.

Extraction and Determination of Proteins

Uninfected and *P. penetrans*-infected nematodes harvested at each interval after inoculation, and mature endospores (2×10^6 spores/10 µl PBS, pH 7.2) used as a control, were obtained as described before. Nematodes in 10 µl PBS, pH 7.2 were disrupted with a pestle, and then 30 µl of the extraction solution containing 1.33× UDC (8M urea, 0.04 M dithiothreitol, 0.00665 M CHES buffer, pH 10) was added to each microfuge tube containing the samples. Microfuge tubes were placed into a water bath for 2 hours at 37 °C, and treated with 20 seconds of sonication (Brankson Cleaning Equipment Company, Shelton, CN) every 15 minutes. Extracts were centrifuged at 10,000 × *g* for 5 minutes at room temperature, and aliquots of the supernatant were collected for storage at −20 °C to carry out ELISA and SDS-PAGE analyses. Protein estimation was performed by a micro-protein assay, based on the Bradford's method (Bradford, 1976) according to the manufacturer's instructions (BioRad, Hercules, CA). Standard curves were generated using bovine serum albumin (BSA) (Sigma), and colorimetric measurement was performed at 595 nm (Hewlett Packard 8451A Diode Array spectrophotometer, Palo Alto, CA). The extraction solution containing only urea and CHES buffer pH 9.8 was made previously, divided in 0.5 ml aliquots, and stored at -20 °C in 1.5 ml microtubes (Fisher Scientific), and then dithiothreitol was added to it just before the extraction of proteins.

Monoclonal Antibody

The anti-P-20 IgM monoclonal antibody (IgM MAb) used in this study was raised in mice against whole endospores of *P. penetrans* P-20 strain and purified on a Sephacryl S-300 column (J. F. Preston and J. D. Rice, unpubl.). This monoclonal antibody showed the ability to block attachment of *P. penetrans* (P-20 strain) to the cuticle of *M. arenaria* race 1, and the IC_{50} is 1.3×10^{-10} M. It recognized an epitope shared on several polypeptides separated by SDS-PAGE (Brito et al., 1998; 2000 Charnecki, 1997; Charnecki et al., 1998).

Epitope Quantification by ELISA

Proteins (100 ng/well) extracted from *P. penetrans*-infected nematodes (either 13 infected nematodes harvested at 12 and 16 DAI or 5 infected nematodes harvested or 24 and 38 DAI) at each harvest interval, or from P-20 strain endospores alone as a positive control (2 x 10^6 endospores/µl), were applied to appropriate wells of a multi-well plate with 100 µl/well of coating buffer (15.00 mM Na_2C0_3, 33.40 mM $NaHC0_3$, and 0.2% NaN_3) added, and incubated overnight at 4 °C. After washing the wells four times with PBST (0.2% Tween 20 in 10 mM sodium phosphate buffer, pH 7.6; 154 mM NaCl), the first antibody, anti-P-20 IgM MAb diluted to 1:100,000 in PBST, was added to the appropriate wells (100 µl/well) and incubated for 1.5 hours at room temperature. Wells were washed with PBST again, and the secondary antibody, anti-mouse IgM-alkaline phosphatase conjugated (Sigma) diluted at 1:4000 in PBST was added to all wells, and incubated for another 1.5 hours at room temperature, and the wells were washed with PBST as before. Alkaline phosphatase substrate, 0.1% *p*-nitrophenol phosphate (w/v)

(Sigma) in alkaline phosphatase substrate buffer (0.05 M Na_2C0_3, 0.05 M $NaHC0_3$, 0.0005 mM $MgCl_2$) was added to all wells, and color development was measured with an automated microplate reader at 405 nm (BioRad model 2550, Hercules, CA).

SDS-PAGE Analysis

Proteins (600 ng of total healthy or infected nematode protein) in an appropriate volume of 10 mM PBS, pH 7.2, were combined with an equal volume of sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS w/v, 10% glycerol, 0.05% bromophenol blue w/v, 2% β-mercaptoethanol), and boiled for 5 minutes at 100 °C, and then centrifuged for 5 minutes at 10,000 × *g*. Endospore protein that was extracted from P-20 isolate (2 x 10^6 endospores/µl) alone was used as a control. Twenty microliters of the supernatants were transferred into appropriate wells of a polyacrylamide gel of 4% stacking gel (pH 6.8) and 12% separating gel (pH 8.8) with Tris-glycine buffer (Laemmli, 1970). A prestained molecular weight marker (SeeBlue ™ Prestained Standards, Novel Experimental Technology, San Diego, CA) was loaded onto the same gel. Electrophoresis was carried out at 100 V for 10 minutes, and then was set at 200 V until the dye marker moved to the bottom of the gel. Gels were electro-blotted onto nitrocellulose membranes in blotting buffer (192 mM glycine, 25 mM Tris, 20% methanol) using a Mini Transfer-blot Cell (BioRad, Hercules, CA) at a constant voltage, 50 V for 2 hours. Proteins either were stained with AuroDye according to the manufacturer's instructions (Amersham, Piscataway, NJ) or with anti-P-20 IgM Mab.

Immunoblotting

The nitrocellulose membranes were blocked with 0.5% non-fat dry milk (w/v) in PBST (10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 0.2% Tween 20) overnight at 4 °C. Polypeptides containing the epitope recognized by anti-P-20 MAb were detected as follows: incubation of the membranes with anti-P-20 IgM MAb at 1: 2,000 in PBST, pH 7.2 for 1.5 hours at room temperature on a shaker, washed three times for 5 minutes each with PBST; incubated with goat anti-mouse IgM MAb conjugated to alkaline phosphatase (Sigma) diluted to 1:1,000 in PBST, pH 7.2 as secondary antibody for 1.5 hours at room temperature on a rotatory shaker, followed by three washes with PBST as above; incubation with substrate buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) three times, five minutes each; incubated with alkaline phosphatase substrate (0.1 mg/ml nitrotetrazolium blue, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate) (Promega, Madison, WI) in substrate buffer on a shaker at room temperature until color development. The blots were washed with deionized water and dried at room temperature.

Immunofluorescence of Whole Endospores

The immunofluorescence staining was performed as described by Pogliano et al. (1985) with modifications. Fresh endospores were washed and purified as before, and then filtered through a woven polyester filter with 18 μm openings. Twenty microliters of the endospore suspension (2×10^6 endospore/μl) were transferred to a 1.5 ml siliconized microtube, and fixed in 230 μl of the primary fixative containing 2.7% formaldehyde and 0.008% glutaraldehyde in 10 mM PBS (10 mM sodium phosphate

buffer, pH 7.4, 150 mM NaCl) for 35 minutes on ice. Endospores were placed in 250 µl 10 mM PBS, pH 7.4 and then centrifuged at 6,000 × *g* three times for 6 minutes each. After resuspending the endospores in 150 µl PBS, 10 µl of the suspension was transferred into each of three wells of a microscope slide which had been treated previously with 0.1% poly-L-lysine (Sigma). Each slide was incubated for 30 seconds at room temperature and then the suspension was aspirated from the wells with a sterile-transfer pipette (Fisher Scientific). After air drying at room temperature for 30 minutes, the endospores were incubated in a 10 µl/well with in PBST-BSA (2% BSA (w/v) and 0.05% Tween 20 (v/v) in 10 mM PBS, pH 7.4) for 15 minutes at room temperature to block nonspecific antibody-binding sites. Primary antibody, anti-P-20 IgM MAb diluted to 1:1,000 in PBST-BSA, was added to the wells and incubated overnight at 4 °C. Wells containing the endospores were washed in PBST, pH 7.4, five times for 5 minutes each, and incubated for 2 hours in the dark at room temperature with micron chain-specific, anti-mouse IgM conjugated with fluorescein isothiocyanate (FITC, Sigma, 1:100 diluted in PBST-BSA). Anti-P-20 IgM MAb was substituted with non-immune ascites fluid at 1:1,000 dilution as negative control. After washing the wells with 10 mM PBS, pH 7.4, 10 times for 5 minutes each, the slides were mounted in Slow Fade in a PBS-glycerol solution (Molecular Probes Inc., Eugene, OR). Preparations were examined with differential-interference contrast and fluorescence microscopy using a Nikon Episcopic Fluorescence attachment with an excitation filter at 495 nm.

Tissue Preparation for Sectioning

Uninfected and *P. penetrans*-infected *M. arenaria* race 1 harvested at 20 days after inoculation at 35 °C were obtained as described above. The procedure used to carry out this study was a modification of the work by Aldrich et al. (1995); Chen et al. (1997a); and Zeikus and Aldrich (1975). Fresh nematodes were ruptured with a surgical knife (Fisher Scientific No. 15) into 40 µl of fixative (1% glutaraldehyde, 4% formaldehyde, 5% dimethyl sulfoxide in 0.1 M sodium cacodylate buffer, pH 7.2) to facilitate the penetration of reagents, and then embedded in 2.5% low temperature gelling agarose (Fisher Scientific) at 45 °C and congealed in the refrigerator (4 °C). The gel was sliced into square blocks containing individual nematodes and transferred into 12 × 75 mm culture tubes (Fisher Scientific) containing 1.5 ml of the above-mentioned fixative, and incubated overnight at 4 °C. Agar blocks containing nematodes were washed four times with cold 0.1 M cacodylate buffer on ice for 30 minutes each and dehydrated in a cold ethanol series containing the following percentages: 12, 25, 38, 50, 65 for 20 minutes each, and then 75 overnight at 4 °C. This was followed by 85, 95 and two changes of 100% ethanol for 20 minutes each. The specimens were embedded in LR White Resin (London Resin White, Electron Microscopy Science, Fort Washington, PA) series (25 and 50% for 3 and 6 hours, respectively, and 75%, 100%, and 100%, overnight each time). Agar blocks containing nematodes were transferred into a 1-ml gelatin capsule containing LR White, and allowed to polymerize at 50 °C for 4 days. Ultrathin sections (50-70 nm thick) were cut from the resin block with a diamond knife on a LKB

8800 Ultratome III microtome (Sweden). Sections were collected on Formvar-coated nickel grids (100 mesh), and processed for immunogold labeling.

Immunogold Labeling

Nickel grids with sections of uninfected and *P. penetrans*-infected nematodes, and with endospore-attached juveniles were floated, section-side down, on 20-µl drops of 1% non-fat dry milk in PBS, pH 7.2 (0.01M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2) on a piece of Parafilm (American National Can ™, Menasha, WI) for 15 minutes at room temperature to block nonspecific antibody-binding sites (modified from Aldrich et al. 1992, 1995; Dykstra, 1993). Grids were floated on 20-µl drops of primary antibody, anti-P-20 IgM MAb at 1:10,000 dilution in PBS, pH 7.2, and incubated overnight in a closed petri dish inside a moist chamber at 4 °C. Control grids were floated on non-immune ascites fluid at 1:10,000 dilution instead of anti-P-20 IgM MAb. Grids were removed, and floated on 20-µl drops of high salt-Tween buffer, pH 7.2 (0.1% Tween 20 in 0.02 M Tris-HCl, pH 7.2, 0.5 M Na Cl), two times for 10 minutes each, and then PBS, pH 7.2, two times for 10 minutes each. Sections were incubated with the secondary antibody, goat anti-mouse IgM conjugated to 12-nm colloidal gold particle, µ-chain specific (Jackson Immuno Research, West Grove, Pennsylvania), diluted 1:30 in PBS, pH 7.2, at room temperature for 1 hour. After washing as above in high salt-Tween buffer and PBS, the grids were floated in Trumps buffer, pH 7.2 (McDowell and Trump, 1976) for 10 minutes at room temperature in order to stabilize the antigen-antibody complex, and then washed with deionized water. Sections were stained with 0.5% uranyl

acetate for 7 minutes, and aqueous lead citrate solution for 2.5 minutes and observed on a Zeiss EM-10 transmission electron microscope at 80 kV. All reagents used to carry out this study were ultrapure-TEM grade.

Results

Microscopic Examination

The vegetative growth stage of *P. penetrans* was observed only in nematodes harvested at 12 and 16 days after inoculation (Table 2.1). At 24 days after inoculation, mixed developmental stages of thalli showed advanced differentiation, including quintets, quartets, triplets, doublets; sporulation, oval-shaped immature sporangium; and mature endospores with visible exosporium were first observed. At 38 days only various phases of sporulation and mature endospores were present in the pseudocoelom of *M. arenaria* race 1.

Epitope Quantification by ELISA

The anti-P-20 IgM MAb did not recognize proteins extracted from infected nematodes harvested at 12 and 16 days after inoculation (Fig. 2.1A). However, the monoclonal antibody reacted with proteins extracted from infected nematodes harvested at 24 and 38 days after inoculation (Fig. 2.1A). The protein per infected nematode was 0.453 µg at 12; 0.466 µg at 16, 1.175 µg at 24, and 2.049 µg/nematode at 38 days after inoculation (Fig. 2.1B). The total protein per infected nematode increased with developmental time (Fig. 2.1B), and was correlated with the increase in the signal detected by the anti-P-20 IgM MAb (Fig. 2.1A). At 24 and 38 days after inoculation, the ELISA-based absorbance at 405 µm per infected nematode was 1.50 and 3.20,

Table 2.1. Percentage of different developmental stages of *Pasteuria penetrans* in *Meloidogyne arenaria* race 1 on tomato 'Rutgers' at 12, 16, 24, and 38 days after inoculation at 35 °C[a].

	Days Postinoculation			
Developmental stage	12	16	24	38
Vegetative growth	90	90	0	0
Differentiation	0	0	15	0
Sporulation	0	0	85	5
Mature endospores	0	0	65	95

[a]Twenty nematodes were observed at each harvest date, and percentage of nematodes at 12, 16, 24, and 38 days after inoculation. Nematodes were hand-picked, placed on a glass slides, and crushed separately in 2.5 µl of lactophenol plus 1% methyl blue (w/v) under a cover glass. Infected nematodes were examined with the use of an inverted microscope (×400) to determine the percentage of the different developmental stages of *P. penetrans* within the pseudocoelom of *Meloidogyne arenaria* race 1. Note that at 24 days after inoculation more than one developmental stage was observed within the pseudocoelom of a single nematode. The developmental stages observed were: vegetative growth including mycelial colonies only within the pseudocoelom; differentiation stage, with presence of thalli differentiation, including quintets, quartets, triplets, doublets; sporulation stage, with many doublets and developing endospore with distal swollen ends connected by intercalary ends; and mature endospores; with free endospores with exosporium clearly visible.

respectively, which was proportional to the amount of adhesin-associated epitope increased as *P. penetrans* reached its maturation stage (Fig. 2.1A). These results suggest that the antigens bearing the epitope, which was recognized by anti-P-20 IgM MAb, were synthesized at later stages of development associated with sporulation of *P. penetrans* within the pseudocoelom of *M. arenaria* race 1.

SDS-PAGE Analysis and Immunoblotting

Analysis of individual proteins extracted from uninfected and *P. penetrans*-infected nematodes at each window of development showed some differences in the protein profiles related to the infection of the nematode by the bacterium (Figs. 2.2A-B; 2.3A-B). The immunoblot showed that Anti-P-20 IgM MAb did not recognize any protein extracted from uninfected nematodes harvested at 12, 16, 24, and 38 days after inoculation (Lanes 2, 3, 4, and 5) (Fig. 2.2B); nor were proteins extracted from infected nematodes harvested at 12 and 16 days detected in the immunoblot (Lanes 2, and 3) (Fig. 2.3B). However the immunoblot revealed that the monoclonal antibody reacted with protein extracts of infected nematodes harvested at 24 and 38 days after inoculation (Lanes 4, 5) (Fig. 2.3B) and with endospore protein of the P-20 strain used as the control (Lanes 6) (Figs. 2.2B; 2.3B).

Immunofluorescence

Note the general shape of P-20 strain just before it was examined with the fluorescence microscope (Fig. 2.4A). Labeling of whole endospores of *P. penetrans*

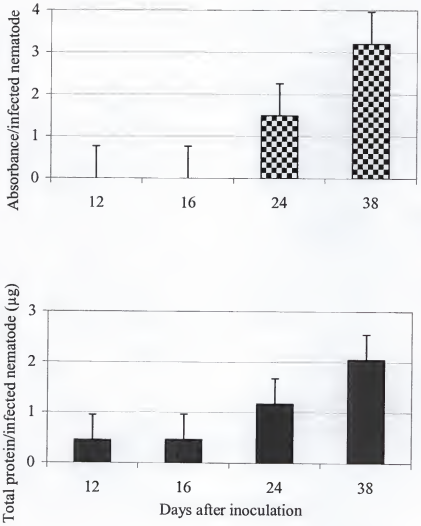


Fig. 2.1. Adhesin-associated epitope and total nematode protein per infected nematode as a function of the development of *Pasteuria penetrans*. A) Levels of adhesin-associated epitope determined by ELISA using anti-P-20 IgM MAb at 1:100,000 dilution in PBST, pH 7.6. Infected nematode total proteins (100 ng/well) was applied in 100 µl/well at the final treatment. Alkaline phosphatase substrate, 0.1% *p*-nitrophenol phosphate (w/v) was added to all wells, and color development was measured at 405 nm. B) Total nematode protein of infected nematodes. Data shown are 40 minutes readings. Lines above the bars indicate SE of the mean for six replicates per treatment.

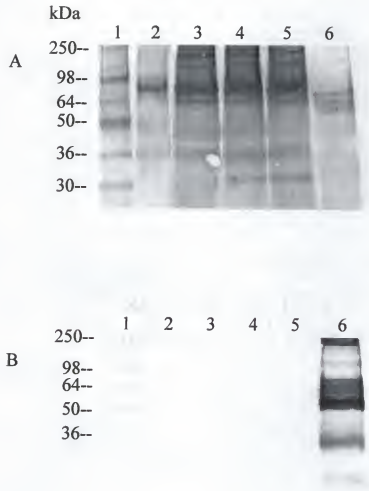


Fig. 2.2. Blots of sodium dodecyl sulfate-polyacrylamide gels of uninfected *Meloidogyne arenaria* protein extracts after electrophoresis. Proteins of uninfected nematodes, harvested at each window of development. Extracts in the appropriate volume of sample buffer were boiled for 5 minutes at 100 °C, and 20 µl of the appropriate extract containing 600 ng of total protein was applied per lane. A) Proteins were detected by staining with AuroDye according to manufacturer's instructions. B) Immunodetection of blotted antigens with anti P-20 IgM MAb at 1: 2,000 dilution in PBST, pH 7.2. Lane 1 - Molecular weight markers, See Blue pre-stained proteins; Lanes 2, 3, 4, and 5 - Total proteins extracted from uninfected nematodes at 12, 16, 24, and 38 days after inoculation; Lane 6 - Proteins extracted from *P. penetrans* P-20 endospores.

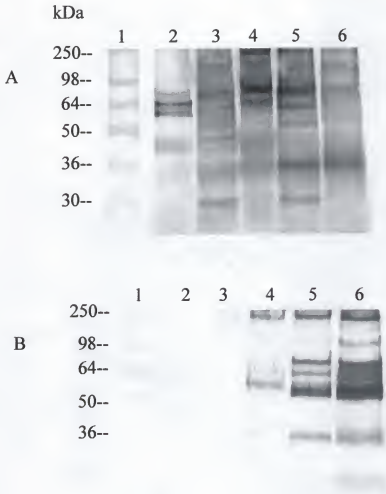


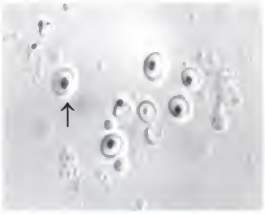
Fig. 2.3. Detection of *Pasteuria penetrans* adhesin-associated epitope as a function of its development within the pseudocoelom of *Melodogyne arenaria* race1. Nematode total proteins and endospore proteins were extracted as for Fig 2. Proteins, 600 ng in 20 µl of the appropriate extract plus sample buffer was loaded into each lane. A) Detection of blotted proteins with AuroDye. B) Western blot of *P. penetrans* infected nematodes probed with anti-P-20 IgM MAb at 1:2,000 dilution in PBST, pH 7.2. Lane 1 - Molecular weight markers, See Blue pre-stained proteins; Lanes 2, 3, 4, and 5 - Epitope bearing proteins extracted from *P. penetrans* infected nematodes at 12, 16, 24, and 38 days after inoculation; Lane 6 - Proteins extracted from *P. penetrans* P-20 endospores.

isolate P-20 by anti-P-20 IgM MAb was not uniform (Fig. 2.4B), which suggests that the adhesin-associated epitope is not uniformly distributed on the surface of mature endospores.

Immunogold Labeling

Anti-P-20 IgM MAb did not recognize any nematode tissue and there were no gold particles observed over the thin section of either uninfected females or J2 with associated endospores (Fig 2.5). The adhesin-associated epitope was not present in the ultrathin sections of vegetative cells (vc) or stage I (Fig. 2.6A) or in stage II of sporogenesis of *P. penetrans* isolate P-20 (Fig. 2.6B). Note a membrane (arrow head) is forming at 1/3 from the anterior, which occurs at this stage of sporogenesis (Chen et al., 1997b). Labeling of the adhesin-associated epitope was first observed over an ultrathin section of the stage III sporogenesis, mainly on the parasporal fibers (pf) (Fig. 2.7A). The antigens bearing the epitope were detected not only over the parasporal fiber (pf) (Figs. 2.7B-2.9A) but also over the sporangium(s) as *P. penetrans* continues to sporulate (Figs. 2.8A-B; 2.9A). The mature endospore was heavily labeled, and the epitope was localized in the sporangium (s), exosporium (ex), and parasporal fibers (pf) (Fig. 2.9A). The outer spore coat (oc), inner spore coat (ic), cortex (c), protoplasm (p), and basal ring (br) were not labeled (Fig. 2.9A). No labeling was observed over any structure of the mature endospore when non-immune ascites fluid was used (Fig. 2.9B).

A



B

Fig. 2.4. Differential interference contrast (DIC) and fluorescence microscopy photomicrographs of whole endospores of *Pasteuria penetrans* P-20 isolate (100× magnification). A) Overall shape of whole endospores using DIC. B) Labeling of an adhesin-associated epitope on the surface of whole endospores using anti-P-20 IgM MAb at 1:1000 dilution in PBST-BSA, overnight at 4 °C, as primary antibody, and anti IgM Mab-FITC labeled as secondary antibody diluted 1:1000 in PBST-BSA. Arrows heads identify regions of nonuniform labeling.

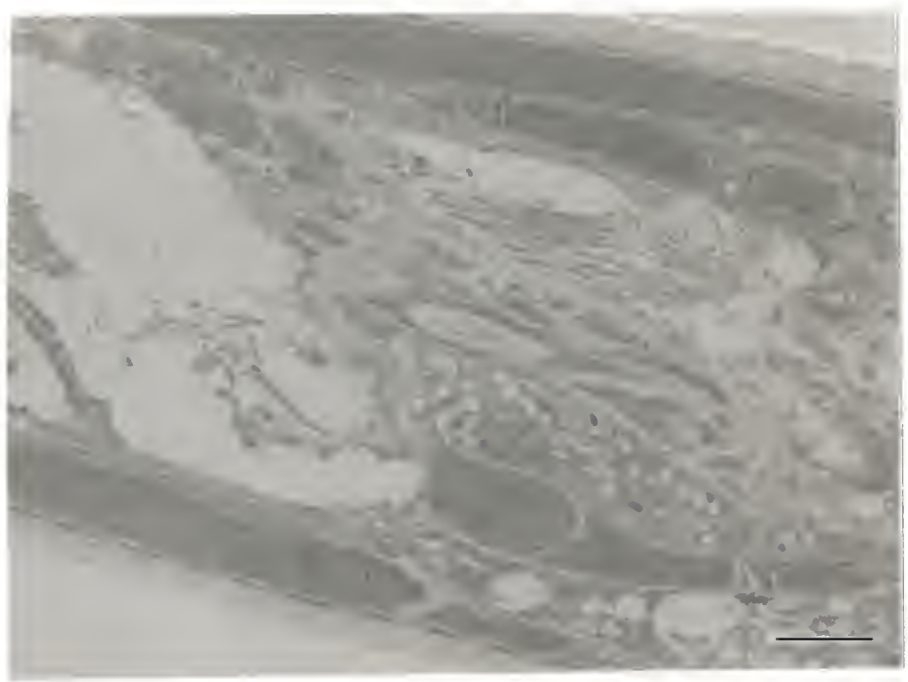


Fig 2.5. Longitudinal section of uninfected second-stage juvenile of *Meloidogyne arenaria* (1-day-old) probed with anti-P-20 IgM MAb at 1:10,000 dilution, and anti-IgM, gold-conjugated at 1:30 dilution. No gold particles are visible over the nematode tissues. Scale Bar = 0.5 µm.

Fig. 2.6. Immunocytochemical localization of an adhesin-associated epitope during the development of *Pasteuria penetrans* within the pseudocoelom of *M. arenaria.* Thin sections of all stages of development of *P. penetrans* were probed with anti- P-20 IgM MAb at 1:10,000 dilution, and anti-IgM MAb gold-conjugated diluted to 1:30 dilution as secondary antibody and examined by transmission electron microscopy. Scale Bars = 0.5 μm. A) Stage I of sporogenesis. A longitudinal ultrathin section of mycelial colony (arrow) of *P. penetrans* P-20 isolate. No labeling is visible over the mycelium. B) Stage II sporogenesis of *P. penetrans.* Note that a membrane is forming at 1/3 distance from the anterior end (arrow read), which is characteristic of this stage. No labeling occurs over any structure of this stage of development of the bacterium.

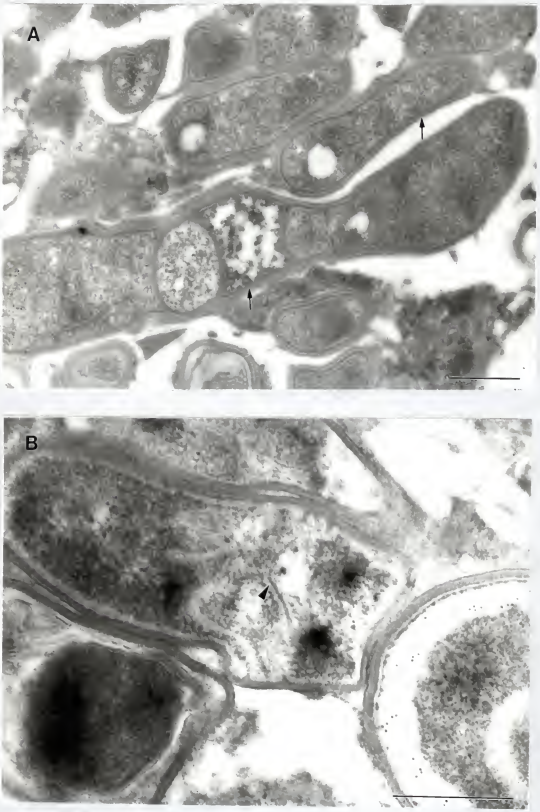

A
B

Fig 2.7. Labeling of sporogenous stages of *Pasteuria penetrans*. Scale bars = 0.5 μm. A) Stage III sporogenesis showing labeling of the adhesin-associated epitope (arrow head) mainly over the parasporal fibers (pf). B) Stage IV sporogenesis, gold particles (arrow head) are concentrate in the parasporal fibers (pf). Note that the vegetative cell (vc) was not labeled.

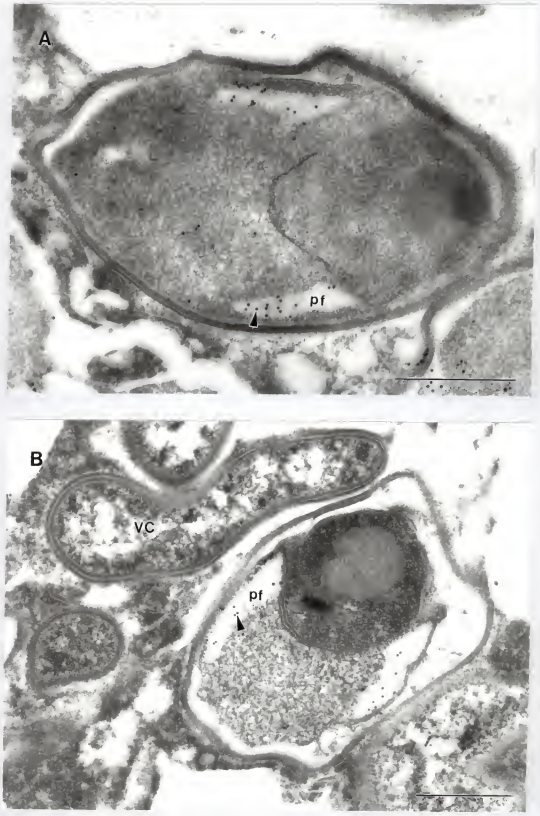

A
pf
B
VC
pf

Fig 2.8. Sporogenous stages of *Pasteuria penetrans*. Scale bars = 0.5 µm. A) Stage V of sporogenesis. Gold label (arrow head) indicating antibody binding is present over the parasporal fibers (pf) and exosporium (e). B) Stage VI of sporogenesis, labeling of the adhesin-associated epitope is observed over the parasporal fibers (pf) and exosporium (ex).

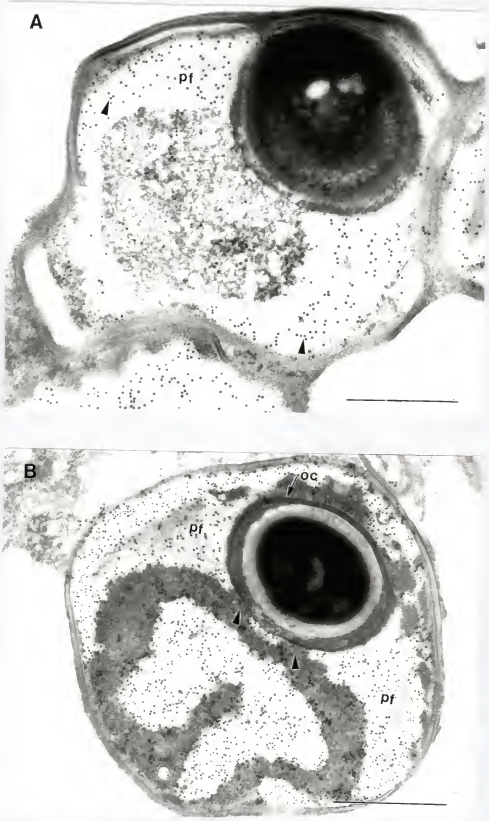
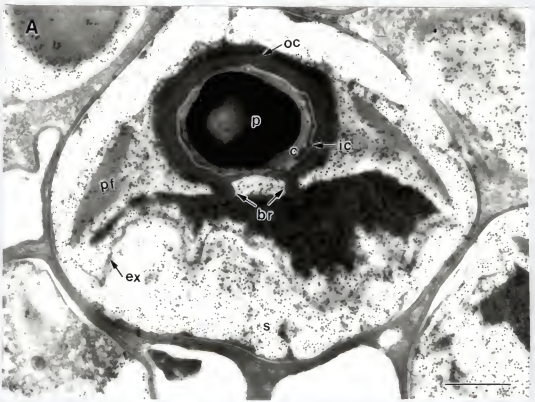

A
pf
B
oc
pf
pf

Fig. 2.9. Late sporogenous stage of *Pasteuria penetrans*. Scale Bars = 0.5 µm. A) Stage VII of sporogenesis, a mature endospore showing the sporangium (s) exosporium(ex), and parasporal fibers (pf) heavily labeled, whereas the outer spore coat (oc), inner spore coat (ic), epicortex (ep), cortex (c), protoplasm (p), and basal ring (br) are not labeled. Note that the parasporal fibers (pf) were not uniformly labeled (arrow head). B) A mature endospore of the *Pasteuria penetrans*, stage VII used as control. No label is observed over the thin section of the endospore.


A
oc
p
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ic
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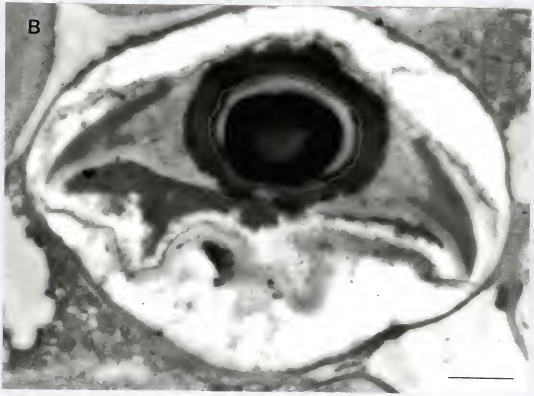
s


B

Discussion

Pasteuria penetrans completes its life cycle within the pseudocoelom of female of root-knot nematodes. The physiological aspects of its life cycle have been studied and are reasonably well understood (Chen and Dickson, 1997; Freitas et al., 1997; Hatz and Dickson, 1992; Serracin et al., 1997; Nakasono et al. 1993, Stirling, 1981, Giannakou et al., 1999). However the biochemical aspects are poorly understood. Seven morphological stages of development through sporulation have been determined as I, II, II, IV, VI, and VII (Chen and Dickson, 1997). The initial step in the life cycle of *Pasteuria* is the recognition/attachment of the endospores to the cuticle of a free living J2 root knot-nematode host. Infection of the host and germination of the endospores occur once the J2 enters the root tissue of a plant host, and establishes a permanent feeding site (Sayre and Starr, 1985, 1988). Vegetative growth, differentiation, and formation of Imbriani, 1975; Sayre, 1993; Sayre and Wergin, 1997; Serracin et al., 1997). The mechanisms involved in the attachment have been the subject of study in several laboratories. The results of these studies have led to the establishment of a model where glycoproteins, designated as adhesins and lectin are involved in the interaction of *P. penetrans* and the nematode host (Persidis et al., 1991; Davies and Danks, 1993). Previous studies have shown that microbial adhesins, or bacterial surface proteins, known as virulence factors such as pili, or fimbriae (Robins-Browne et al., 1994; Salyers and Whitt, 1994; Suoniemi et al., 1995), and afimbrial adhesins (Salyers and Whitt, 1994), allow bacteria to attach, colonize, and invade their hosts. For instance, *Streptococcus pyogenes*, a gram-positive pathogen has a nonfibrillar adhesin (protein F) that mediates its

attachment to fibronectin, a protein found on many host cell surfaces, including the mucosa of the human throat (Salyers and Whitt, 1994). However, the mechanisms used by *Pasteuria* spp. to produce virulent endospores within the pseudocoelom of the nematode host is not well understood. Mohan et al. (2001) found that fibronectin-like proteins extracted from *M. javanica* are involved in the attachment of endospores. In this study, we determined the relative time of the synthesis of an adhesin-associated epitope during the development of *P. penetrans* within the pseudocoelom of *M. arenaria* race 2; detected and localized this epitope during endospore development, and also determined the distribution of the epitope on the surface of mature endospores using a monoclonal antibody directly selected against whole mature enendospores of *P. penetrans* P-20 isolate.

ELISA and immunoblot analysis revealed that only proteins extracted from *P. penetrans*-infected nematodes at 24 and 38 days after inoculation were recognized by anti- P-20 IgM MAb and the amount of the epitope was highest at the height of sporulation (38 days after inoculation) than at any other developmental stage (12, 16, and 24 days after inoculation). The Western blot showed a higher degree of similarity in the protein profile of *P. penetrans*-infected nematodes at 38 days after inoculation to the mature P-20 spore protein, used as a control, than with *P. penetrans*-infected nematodes from any other window of development. Examination of the infected nematodes harvested at 12 and 16 days by light microscopy revealed that only the vegetative growth stage, including clusters of mycelial colonies and thalli, were found throughout the pseudocoelom of nematodes. At 24 and 38 days, sporulation and maturation stages were

observed within the pseudocoelom. Therefore, the synthesis of the adhesin-associated epitope occurred at a certain developmental stage relative to the sporogenesis process, and it was absent in vegetative growth and differentiation stages.

The synthesis of specific molecules at specific times during the germination, growth, and sporulation of the endospore-forming bacterium, *Bacillus subtilis*, has been rigorously established. For instance dipicolinic acid (pyridine-2, 6-dicarbonate) is formed during the first 5 hours of sporulation (Schlegel, 1986), whereas the small, acid-soluble spore proteins (SASPs), a group DNA-binding proteins (at neutral to slightly alkaline pH), are synthesized after 3-4 hours into sporulation (Johnson and Tipper, 1981; Setlow 1985). Both molecules are found only in endospores (Fliss et al., 1985; Schlegel, 1986). Even though some molecules are synthesized at specific stages of sporulation, it is possible that they are degraded and used to carry out a certain function at another stage. For instance, during the first 5 hours of sporulation in *B. subtilis* much of the vegetative cell protein is degraded (Schlegel, 1986).

Immunofluorescence labeling showed that the adhesin-associated epitope is not uniformly distributed on the surface of virulent endospores. The heterogeneity of endospore surface has been observed not only within populations but also between populations of *P. penetrans* (Davies and Redden, 1997). Previous studies have shown that differences in the amount and nature of spore-surface proteins, as recognized by several monoclonal antibodies, may account for surface heterogeneity of endospores as well as host specificity (Davies et al., 1992). Davies et al. (1994) using monoclonal antibodies showed that the surface of endospores of the PP1 strain of *P. penetrans* is

highly heterogenous. These and subsequent studies (Davies and Redden, 1997) have suggested that endospore surface properties are responsible for the virulence of *P. penetrans*.

Antigens bearing the epitope were synthesized during the sporogenesis process. Labeling was first observed at stage III of the sporogenesis, mainly in the parasporal fibers. In contrast to stage III sporogenesis, mature endospores were heavily labeled and the adhesin-associated epitope was localized in the parasporal fibers, sporangium, and exosporium.

The general pattern of the labeling of the adhesin-associated epitope over thin sections of a mature endospore was similar to a previous study, where mature endospores were probed with a polyclonal antibody (Persidis et al., 1991). These authors concluded that the labeling did not show any preference to a certain structure of the endospore and suggested that a nonspecific binding of the antibodies could have occurred. These observations may reflect a heterogeneity in the polyclonal antibody preparation and/or selection of a single stage of development. In our immunocytochemistry work, it was shown that the adhesin-associated epitope is synthesized at a certain stage of development related to endospore formation and it is localized initially in the parasporal fibers early in stage III, becoming widespread throughout the sporangium and exosporium, but not in the central body of the stages IV, V, VI, and VI of sporogenesis. Label was not uniformly distributed in the parasporal fibers. Also no labeling was observed in the outer or inner spore coat, epicortex, cortex, protoplasm, and basal ring.

These observations establish a window of development in which the adhesin-associated epitope is formed, and where further studies concerning the formation of this epitope should be directed. The fact that the epitope is distributed over several structures of the mature endospores suggests its involvement in the recognition of the nematode host as an early event in the attachment process. It may increase the chances for a cooperative interaction between the adhesin epitope with receptors on the cuticle of the nematode host, such as carbohydrate binding proteins (Bird et al., 1989; Davies and Danks, 1993; Persidis et al., 1991) and fibronectin-like residues. (Mohan et al., 2001) as well as other forces, that may be involved in the attachment, such as hydrophobic interactions (Afolabi et al., 1995; Davies et al., 1996; Esnard et al., 1997).

CHAPTER 3
DETECTION OF ADHESIN PROTEINS AND IMMUNOLOGICAL DIFFERENTIATION OF *Pasteuria* spp. USING A MONOCLONAL ANTIBODY

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr, the first species of *Pasteuria* described as a parasite of plant-parasitic nematodes, is a widespread endospore-forming bacterial parasite of root-knot nematodes (*Meloidogyne* spp.) (Sayre and Starr, 1985). Over the years, several more species of nematodes in other genera have been reported as hosts of species of *Pasteuria*. (Chen and Dickson, 1998). To date, three species of *Pasteuria* have been described in addition to *Pasteuria penetrans* (Sayre and Starr, 1985). These are *Pasteuria ramosa*, a parasite of water fleas, *Daphnia* spp. (Sayre et al., 1983) which is the type species of the genus; *Pasteuria thornei* isolated from *Pratylenchus* spp. (Starr and Sayre, 1988), and *Pasteuria nishizawae* a parasite of cyst nematodes of the genera *Heterodera* and *Globodera* (Sayre et al.,1991). In recent years more species of *Pasteuria* have been proposed: i) *Pasteuria sp.*, designated as S-1 strain (Bekal et al., 2000) from *Belonolaimus longicaudatus* Rau; ii) a large- and a small-spored isolate of *Pasteuria* spp. each from *Hoplolaimus galeatus* (Cobb) Thorne (Giblin-Davis et al., 1990); and iii) three isolates which attach and complete their life-cycles in *Heterodera* spp.; one isolate was from cereal cyst nematode, *Heterodera avenae* Wollenweber (Davies et al., 1990), a second strain from pea cyst nematode, *Heterodera goettingiana*

Liebscher in Münster, Germany (Sturhan et al., 1994), and a third isolate that infects soybean cyst nematode, *Heterodera glycines* Ichinohe, *Pasteuria* sp. NA (*Heterodera glycines*-infecting *Pasteuria*), Urbana, IL, USA (Atibalentja et al., 2000).

Traditionally species of *Pasteuria* are identified based on morphometrics, morphology, ultrastructural characteristics, and host specificity (Davies et al., 1990; Giblin-Davis et al., 1995; Sayre and Starr, 1985; Sayre et al., 1983; 1991; Starr and Sayre, 1988; Sturhan et al., 1994). More recently, 16S rDNA has been used to carry out systematics studies of *P. ramosa* (Ebert et al., 1996), *P. penetrans* (Anderson et al., 1999), *Heterodera glycines*-infecting *Pasteuria* (Atibalentja et al., 2000), and *Pasteuria* sp. S-1 strain (Bekal et. al., 2000). Also, the use of serology through hybridoma technology might be a useful probe for the identification of *Pasteuria* spp. The anti-P-20 IgM monoclonal antibody (MAb) raised against whole mature endospores of P-20 isolate of *P. penetrans* was used as a probe in this study. This MAb was selected on the basis of its ability to block attachment of *P. penetrans* isolate P-20 to *M. arenaria* race 1 (Charnecki et al., 1998) (Chapter 2). Previous studies have shown that this MAb recognized an epitope shared on several polypeptides separated by SDS-PAGE (Brito et al., 1998; Charnecki 1997; Charnecki et al., 1998). The appearance of an adhesin-associated epitope was tracked during development and localized during sporogenesis of the P-20 within its nematode host (Brito et al., 1998; 1999). The objectives of this study were to determine whether different strains and species of *Pasteuria* share this adhesin-

associated epitope which is involved in the attachment of *P. penetrans* P-20 strain to *M. arenaria* race 1, and to use anti-P-20 IgM MAb as a probe to separate strains and species of *Pasteuria.*

Material and Methods

Origin of *Pasteuria* Species and Isolates

The designations and origins of the species and isolates of *Pasteuria* spp. (Table 3.1) were as follows: two isolates of *P. penetrans*; one designated P-20 (Oostendorp et al., 1990) originally collected from *M. arenaria* race 1 (Neal) Chitwood, from peanut (*Arachis hypogea* cv. Florunner) roots growing in a naturally infested field in Levy County, FL, and the other one designated P1-UFLA (Souza and Campos, 1997), originally isolated from a mixed population of *M. javanica* and *M. incognita*, Lavras, Minas Gerais, Brazil; *H. glycines*-infecting *Pasteuria*, (*Pasteuria* sp. NA) (Atibalentja et al., 2000) from cysts of *H. glycines* collected from the rhizosphere of soybean plants (*Glycines max* (L). Mirril), Urbana, IL. *Pasteuria* sp. strain S-1 (Bekal, et al., 2001; Giblin-Davis et al., 2001) isolated from the sting nematode *B. longicaudatus*, L-1 (large-spored strain), LS-1 (small- spored strain) from the lance nematode, *H. galeatus* (Giblin-Davis et al., 1990), and *Pasteuria* from *Rhabditis* sp. (Giblin-Davis pers. comm.) were all originally collected from bermudagrass (*Cynodon* spp.) turf growing in a naturally infested field, at the Ft. Lauderdale Research and Education Center, University of Florida, Broward County, Fort Lauderdale, FL. *Pasteuria* sp. C-1 isolate (Han et al., 1999) was originally collected from *Criconemoides* sp. in a naturally infested soil where peanut

(Arachis hypogea L. cv. Florunner) was growing at the Green Acres Agronomy Farm, University of Florida, Alachua County, Gainesville. A ring nematode isolate of *Pasteuria* also isolated from *Criconemoides* sp. collected in a peanut field (Williston), FL (Dickson per. comm.), and spiral nematode isolate of *Pasteuria* isolated from *Helicotylenchus* sp. extracted from the rhizosphera of bermudagrass turf from California (Crow, pers. comm.).

Propagation of Bacterial Species and Isolates

Pasteuria penetrans P-20 and P1-UFLA isolates were propagated on *M. arenaria* race 1 and *M. javanica* respectively, growing on 'Rutgers' tomato. Endospores of each strain were attached separately to second-stage juveniles (J2) (up to 2 day old) of root-knot nematodes using a centrifugation method (Hewlett and Dickson, 1993). Juveniles (3,000 J2 per plant) with approximately six endospores attached per J2 were inoculated on 55-day-old tomato plants growing in 15-cm-diameter clay pots in a greenhouse. Endospore-filled females were harvested from the root systems 45 to 60 days after inoculation. Root systems were placed in a 1-liter Erlenmeyer flask containing Rapidase Pomaliq 2F (Gist Brocadest Pomaliq, 7003-A/DSM Food Specialities USA Inc., Menominee, WI) optimized previously with a buffer system at 1:5 (g/v) (Charnecki, 1997), and placed on a shaker at 120 oscillations/minute for approximately 24 hours at room temperature. The softened roots and nematodes were poured onto a sieve with 600 µm-pore openings nested in a sieve with 150 µm-pore openings, and subjected to a heavy stream of tap water to dislodge the nematodes (Hussey, 1971), with modifications. Nematodes and root debris were collected in a beaker, and the contents centrifuged in

Table 3.1. Species and isolates of *Pasteuria.*

Species or isolates	Reference
P. penetrans P-20	*Meloidogyne. arenaria* race 1 (Oostendorp et al., 1990)
P. penetrans P1-UFLA	*Meloidogyne spp.* (Souza and Campos, 1997)
Hg *Pasteuria sp.* NA	*Heterodera glycines* (Atibalentja et al., 2000)
Pasteuria sp. S-1	*Belonolaimus longicaudatus* (Bakel et al., 2001)
C-1 isolate	*Criconemoides* sp. (Han et al., 1999)
L-1 isolate	*Hoplolaimus galeatus* (Gibli-Davis, 1990)
LS-1 isolate	*Hoplolaimus galeatus* (Gibli-Davis, 1990)
Rhabditis infecting-*Pasteuria*	*Rhabditis* sp. (Giblin-Davis, pers. comm.)
Ring nematode-infecting *Pasteuria*	*Criconemoides* (Dickson, pers. comm.)
Spiral nematode-infecting *Pasteuria*	*Helicotylenchus* sp. (Crow, pers. comm.)

20% sucrose (w/v) at 1,500 × *g* for 5 minutes, and the resulting pellet was again centrifuged in 47% (w/v) sucrose (Chen et al., 2000). Female nematodes were collected and examined for *Pasteuria* infection with an inverted microscope at 100 × magnification (Leica, Davie, FL). *Pasteuria*-infected females were hand-picked using a dissecting microscope at 40 × magnification (Nikon, Marietta, GA), and placed in 1.5 ml siliconized microtubes containing 900 µl of deionized water. Nematodes were centrifuged in deionized water three times at 10,000 × *g* for 2 minutes, and then stored in 500 µl deionized water at 4 °C until used. *Pasteuria* sp. S-1, L-1, LS-1 isolates, and the *Rhabditis* sp. infecting-*Pasteuira*, and spiral nematode-infecting *Pasteuria* were isolated from their nematode hosts growing in bermudagrass (*Cynodon dactylon* (L) X *C. transvaalensis* Burt-Davy cv. Tifway or *C. magenissii* Hurcombe cv. Tifgreen) turf in a naturally-infested field. The C-1 isolate and ring nematode-infecting *Pasteuria* were obtained from *Criconemoides* sp. extracted from the rhizosphere of peanut (*Arachis hypogea* L. cv. Florunner) grown in a naturally-infested soil in a greenhouse, and peanut field, respectively. All nematodes were extracted from the soil using a centrifugal-flotation method (Jenkins, 1964). *Pasteuria*-infected nematodes were hand-picked under a dissecting microscope, and placed in deionized water. After washing the nematodes with deionized water as above, they were stored in 900 µl deionized water at 4° C until used. *Pasteuria* sp. NA was propagated on *H. glycines* race 3 reared on soybean cv. Lee growing in a naturally-infected soil in a greenhouse. *Pasteuria*-infected cysts and females were extracted from the rhizosphere of 3-month old soybean plants by washing the soil through a sieve with 850 µ-pore openings nested over a sieve with 180 µ-pore openings;

and nematodes were collected in a sterile beaker. Nematodes were transferred into 200-ml centrifuge tubes containing 150 ml of deionized water, and centrifuged at 2,000 × *g* for 4 minutes. The resulting pellets were re-suspended with 50% sucrose solution, and again centrifuged for 35 to 45 seconds. The supernatant was poured through a sieve with 180 µ-pore openings (Atibalentja et al., 2000), and collected in a sterile beaker. Infected females and cysts were hand-picked based on their opaque appearance, washed three times with deionized water by centrifugation at 10,000 × *g* for 2 minutes, placed in a 1.5 ml siliconized microtube containing 100 µl deionized water, and stored at 4 °C until used.

Extraction and Determination of Proteins

Nematodes infected by species or isolates P-20, P1-UFLA, S-1, C-1, ring nematode and spiral nematode isolates of *Pasteuria*, and cysts infected with the *Pasteuria* sp. NA strain were obtained as described before. Infected nematodes and cysts in the appropriate 1.5 ml siliconized microtube containing deionized water were crushed with a pestle, filtered with 18-µm-pore membrane, and the endospore concentration of the suspension was determined with a hemocytometer (Fisher, Suwanee, GA) under a compound microscope (Leica, Davie, FL) at a magnification of 40×. Ten microliters of endospore suspension was transferred to a 1.5 ml siliconized microtube, and 30 µl of the extraction solution containing 1.33× UDC (8 M urea, 0.04 M dithiothreitol,0.00665 M CHES buffer, pH 9.8) was added. Microtubes were placed into a water bath for 2 hours at 37 °C, with 20 seconds of mild sonication (Brankson Cleaning Equipment Company, Shelton, CN), every 15 minutes. Extracts were centrifuged at 10,000 × *g* for 5 minutes at

room temperature, and aliquots of the supernatant were collected and stored at −20 °C until used. Protein estimation was performed by a micro-protein assay, according to the manufacturer's instructions (BioRad, Hercules, CA). Standard curves were generated using bovine serum albumin (BSA) (Sigma, St. Louis, MO), and colorimetric measurement was performed at 595 nm (Hewlett Packard 8451A Diode Array spectrophotometer, Palo Alto, CA). The UDC stock solution was made previously using only urea and CHES buffer, pH 9.8, divided in 0.5 ml aliquots, and stored at -20 °C in 1.5 ml microtubes. Dithiothreitol was added to the microtubes just before the extraction of proteins.

Preparation of Infected Nematodes for TEM

All *Pasteuria*-infected nematodes were obtained as described above except for the NA *Pasteuria* which was obtained as follows: infested dry soil (50 g) was placed in a 100×15 ml petri dishes, and the soil water was adjusted to 100% field capacity to increase the rate of endospore attachment. The dish was left uncovered at room temperature (Brown et al., 1985). After 3 days 1,000 juveniles (J2) of *H. glycines* race 3 were added, and the moisture level was adjusted to 50% of field capacity. Dishes were incubated for 7 days at room temperature (Oostendorp et al., 1990), and the J2 were extracted by the centrifugal-flotation method (Jenkins, 1964). J2 with endospore attached were hand-picked, and placed in a 1. 5 ml microtube, washed three times with deionized water by centrifugation at 10,000 × *g* for 2 minutes, and stored at 4 °C until used.

A modified protocol was used to carry out the TEM part of this study (Aldrich et al., 1995; Chen et al., 1997a; Zeikus and Aldrich, 1975). Nematodes were hand-picked

into a 40 µl-drop of fixative (1% glutaraldehyde, 4% formaldehyde, 5% dimethyl sulfoxide in 0.1 M sodium cacodylate buffer, pH 7.2), and cut into 2 to 4 pieces with a surgical knife (Fisher Scientific No. 15) to aid penetration of the reagents. Nematodes were transferred into a 50 µl-drop of 2.5% agarose (Fisher) at 45°C , and then cooled in a refrigerator. After cutting the gel into square blocks, they were placed in 12×75 millimeter culture tubes (Fisher) containing 1.5 ml of the fixative, and incubated overnight at 4 °C. After rinsing the nematodes four times with 0.2 M cadodylate buffer, pH 7.2 on ice for 30 minutes each, they were dehydrated in a cold ethanol series: 12, 25, 38, 50, 65, 75, 85, 95, and two changes of 100% for 20 minutes each, except for 75%, which was kept overnight at 4 °C. Specimens were infiltrated with LR White resins (London Resins White, Electron Microscopy Science, Fort Washington, PA) series: 25% and 50% for 3 and 6 hours, respectively, 75% and two changes in 100% overnight each time). Blocks were placed in 1ml-gelatin capsule containing LR White resin, and allowed to polymerize for 4 days at 50 °C. Thin sections, 50-70 nm thick were cut with a diamond knife on a LKB 8800 Ultratome III microtome (Sweden). Sections were collected and mounted on Formvar-coated nickel grids (100 mesh) and processed for immunocytochemistry.

Immunocytochemistry

Nickel grids containing section were placed, face down, on 20µl-drops of 1% non-fat dry milk in PBS, pH 7.2 (0.01M sodium phosphate buffer, 0.15 M sodium chloride) on a piece of Parafilm (American National Can, Menasha, WI) for 15 minutes at room temperature, to block nonspecific antibody-binding sites (Aldrich et al., 1992; 1995;

Dykstra, 1993) with modifications. Grids were transferred to 20 µl-drops of the first antibody, anti-P-20 IgM MAb at 1:10,000 or 1:40,000 dilution in PBS, pH 7.2, and incubated overnight in a closed petri dish inside of a moist chamber at 4 °C. Grids were floated in 20 µl-drops of high salt tween buffer, pH 7.2 (0.1% Tween 20 in 0.02M Tris-HCl, pH 7.2, 0.5 M NaCl), and in PBS, pH 7.2 twice in each buffer for 10 minutes each, before incubation with goat anti-mouse IgM conjugated to colloidal gold (1:30 dilution in PBS, pH 7.2, 12 nm gold) (Jackson Immuno Research, West Grove, PA) for 1 hour at room temperature. Grids were washed again in high salt tween buffer, and PBS, and were incubated for 10 minutes in Trumps buffer, pH 7.2 (McDowell, and Trump, 1976) at room temperature in order to stabilize the antigen-antibody complex. Sections were washed with deionized water, and stained with 0.5% uranyl acetate for 7 minutes, and aqueous lead citrate solution for 2.5 minutes. Controls were probed with non-immune ascites fluid and goat-anti mouse IgM conjugated to gold to ensure that the results were not due to non-specific binding. Sections were examined on a Zeiss EM-10 transmission electron microscope at 80kV. All reagents used in this study were ultra pure-TEM grade.

SDS-PAGE Analysis

Proteins extracted from endospores of *Pasteuria* NA, S-1, C-1, P1-UFLA, P-20, ring nematode and spiral nematode isolates of *Pasteuria* were individually combined with equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS w/v, 10% glycerol, 0.05% bromophenol blue w/v, 2% β-mercaptoethanol) (BioRad), boiled for 5 minutes at 100 °C, and centrifuged for 5 minutes at 10,000 × *g*. Twenty microliters (600 ng of protein) of the supernatant was loaded into the wells of a Tris-glycine polyacrylamide 4%

stacking gel (pH 6.80) and 12% separating gel (pH 8.8) (BioRad). Electrophoresis was carried out at 100 V for 10 minutes, and then it was set for 200V until the bromophenol blue dye had migrated to the bottom of the gel. Proteins were transferred onto nitrocellulose membranes in blotting buffer (192 mM glycine, 25 mM Tris, 20% methanol) using a Mini Transfer-blot Cell (BioRad) at a constant voltage, 50 V for 2 hours. Protein bands were visualized either by Aurodye (Amersham, Piscataway, NJ) according to manufacturer's instructions or anti-P-20 IgM MAb (Chapter 2). Standard ladders for molecular mass were loaded in the same gels (SeeBlue ™ Prestained Standards, Novel Experimental Technology, San Diego, CA).

Immunoblotting

Blots were first blocked overnight with 0.5% skimmed milk (w/v) in PBST (10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 0.2% [v/v] Tween 20 at 4 °C. Blots then were incubated with anti-P-20 IgM MAb diluted 1:2,000 in PBST, pH 7.2 for 1.5 hours on a rotatory shaker at room temperature, and washed with PBST, three times, 5 minutes each. Blots were incubated with goat-anti mouse IgM conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in PBST, pH 7.2 for 1.5 hours at room temperature, and washed as before with PBST, pH 7.2. After washing blots with substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) three times, 5 minutes each at room temperature, blots were incubated with alkaline phosphatase substrate (0.1 mg/ml nitrotetrazolium blue, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate) (Promega, Madison, WI) in substrate buffer on a rotatory shaker at room temperature until color development. Blots were washed with deionized water and dried at room temperature.

Results

Immunocytochemistry

Intense gold labeling was specifically associated with sporangium (s), exosporium (ex), and parasporal fibers (pf) of P-20, P1-UFLA, *Rhabditis*-infecting *Pasteuria,* S-1, LS-1, L-1, and C- 1 (Figs. 3.1-4). Labeling was not observed over the outer spore coat (oc), inner spore coat (ic), cortex (c) (Figs. 3.1-4), and basal ring (br) (Figs. 3.1A, B) of the endospores of P-20 and P1-UFLA, collected in USA and Brazil. No labeling was observed over any structure of *Pasteuria* sp. NA used as a control (Fig. 3.5). Gold particles were not observed on the germ tube (gt) of *Pasteuria* sp. NA, nor over the cuticle of the cyst nematode, *H. glycines* (Fig. 3.6), however the parasporal fibers (pf) were labeled heavily (Fig. 3.6).

SDS-PAGE and Immunoblotting Analysis

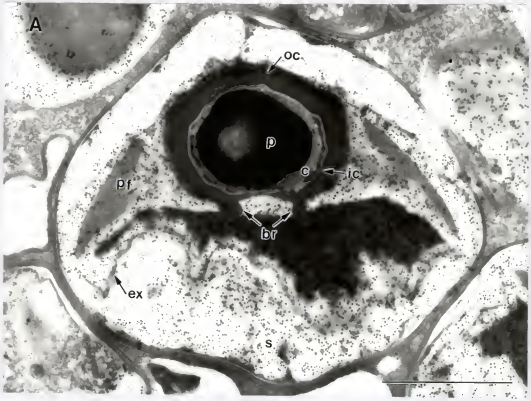
AuroDye staining showed that at least three bands of proteins (arrow head) are common among the *Pasteuria* sp. NA, S-1, C-1, P1-UFLA, ring nematode and spiral nematode isolate of *Pasteuria* and P-20, used as control (Fig 3.7A). Immunoblotting showed qualitative and quantitative differences among all the those isolates and species of *Pasteuira* (Fig 3.7B). All species and isolates share the same epitope because it was recognized by anti-P-20 IgM MAb (Lanes 2-8) (Fig.3.7B). Isolates P1-UFLA and P-20 showed similar bands of proteins with equal intensity (Lane 5 and 6) (Fig. 3.7B). Similarities in bands of proteins also were observed between spiral nematode isolate of *Pasteuria* and ring nematode isolate of *Pasteuria* (Lanes 7 and 8) (Fig. 3.7B). Also the same degree of similarity in the protein profiles was observed among the *Pasteuria* sp.

NA, P1-UFLA, and P-20 extracts (Lanes 2, 5,and 6) (Fig. 3.7B). The strongest bands were observed in proteins extracts from *Pasteuria* sp. NA, P1-UFLA, and P-20 (Lanes 2, 5, and 6) (Fig. 3.7B). *Pasteuria* sp. S-1 showed one band of protein (arrow) (Lane 3) (Fig. 3.7B) that is shared among all other strains (Lanes 2, 4, 5, 6, 7, and 8) whereas C-1 strain showed one band of protein (arrow) (Lane 4) (Fig. 3.7B) that is also observed from the protein extract of *Pasteuria* sp. NA, P-20, and ring nematode-infecting *Pasteuria* (Lanes 2, 6, and 8). The isolate C-1 showed one strong band of protein with molecular weight between 50 and 36 kDa (Lane 4), which appeared similar to a band of less intensity from the extract of the spiral nematode infecting-*Pasteuria* (Lane 7) and ring nematode-infecting *Pasteuria* extract (Lane 8) (Fig. 3.7B).

Discussion

The immunocytochemistry indicated that the adhesin-associated epitope as recognized by anti-P-20 IgM MAb is shared among P-20, P-1 UFLA, NA *Pasteuria* strain, *Rhabditis* sp.-infecting *Pasteuria*, *Pasteuria* sp. S-1 strain, C-1, LS-1, and L-1. The immuno-gold labeling patterns were similar for all the species and isolates examined. The broad distribution of the adhesin epitope over several structures of endospores of different species and isolates of *Pasteuria* may increase their capabilities to attach to their host due to cooperative interactions between the adhesin epitope with receptors on the cuticle of the nematode host, such as carbohydrate binding proteins (Bird et al., 1989; Davies and Danks, 1993; Persidis et al., 1991) and fibronectin-like residues (Mohan et al.,

Fig.3.1. Transmission electron micrographs of *Pasteuria* endospore sections, probed with anti-P-20 IgM MAb at 10,000 dilution. Scale bars = 1 μm. A) Thin section of an mature endospore of P-20 isolate. Labeling was observed over the sporangium (s) exosporium (ex), and parasporal fibers (pf), whereas the outer spore coat (oc), inner spore coat (ic), cortex (c), and basal ring (br) were not labeled. Note that the parasporal fibers (pf) were not uniformly labeled (arrow head). B) Section of a mature endospore of P1-UFLA. Sporangium (s), exosporium(e), and parasporal fibers (pf) are heavily labeled. Labeling is absent over the outer spore coat (o), inner spore coat (i), and basal ring (br).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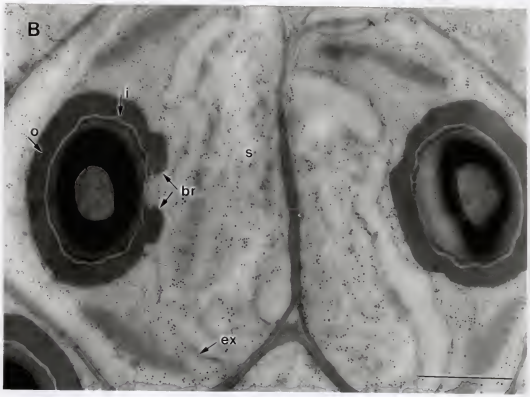
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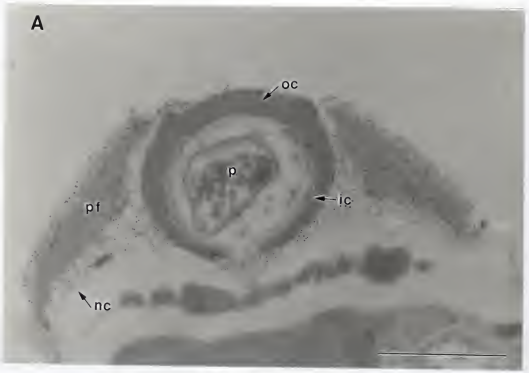
Fig. 3.2. Gold labeling of endospores of different isolates and species of *Pasteuria*. Scale bars = 1 μm. A) Thin section of a mature endospore of the *Pasteuria* sp. NA attached to a second-stage juvenile showing the labeling of the adhesin-associated epitope over parasporal fibers (pf), but not on the outer spore coat (oc), inner spore coat (ic), protoplasm (p), and nematode cuticle (nc). B) Endospore of *Rhabditis* sp.-infecting *Pasteuria* showing the labeling the adhesin-associated epitope over the sporangium (s), and parasporal fibers (pf). No labeling is observed over the vegetative cell (vc).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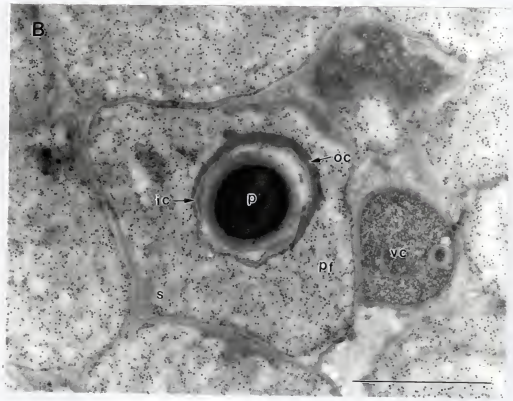
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Fig. 3.3. Immunoelectron microscopy of endospores of *Pasteuria* spp. A) Labeling of an adhesin-associated epitope over a thin section of a endospore of S-1 strain. Gold particles (gp) are observed on the sporangium (s), exosporium (ex), and parasporal fibers (pf), but not over the outer spore coat (oc) and, inner spore coat (ic). Scale bar = 1 μm. B) Thin section of a endospore of the C-1 isolate showing gold particles (gp) over the sporangium (s), exosporium(ex), and parasporal fibers (pf). Scale bar = 0.5 μm.

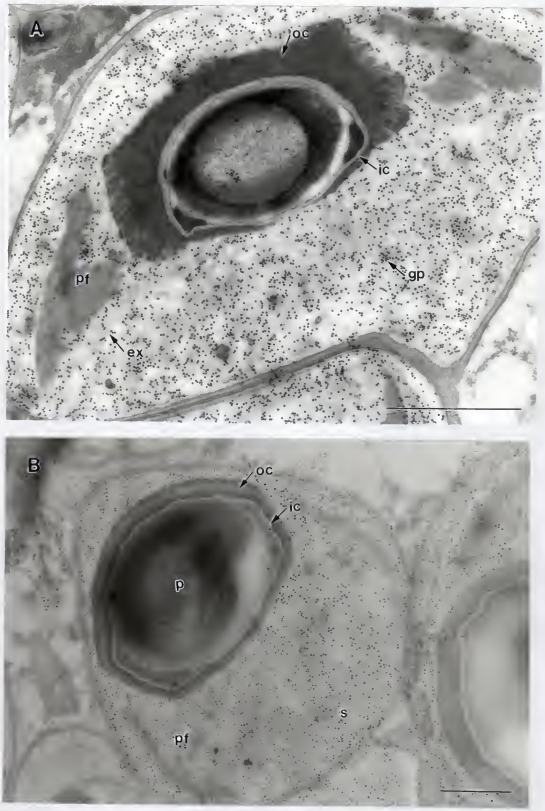
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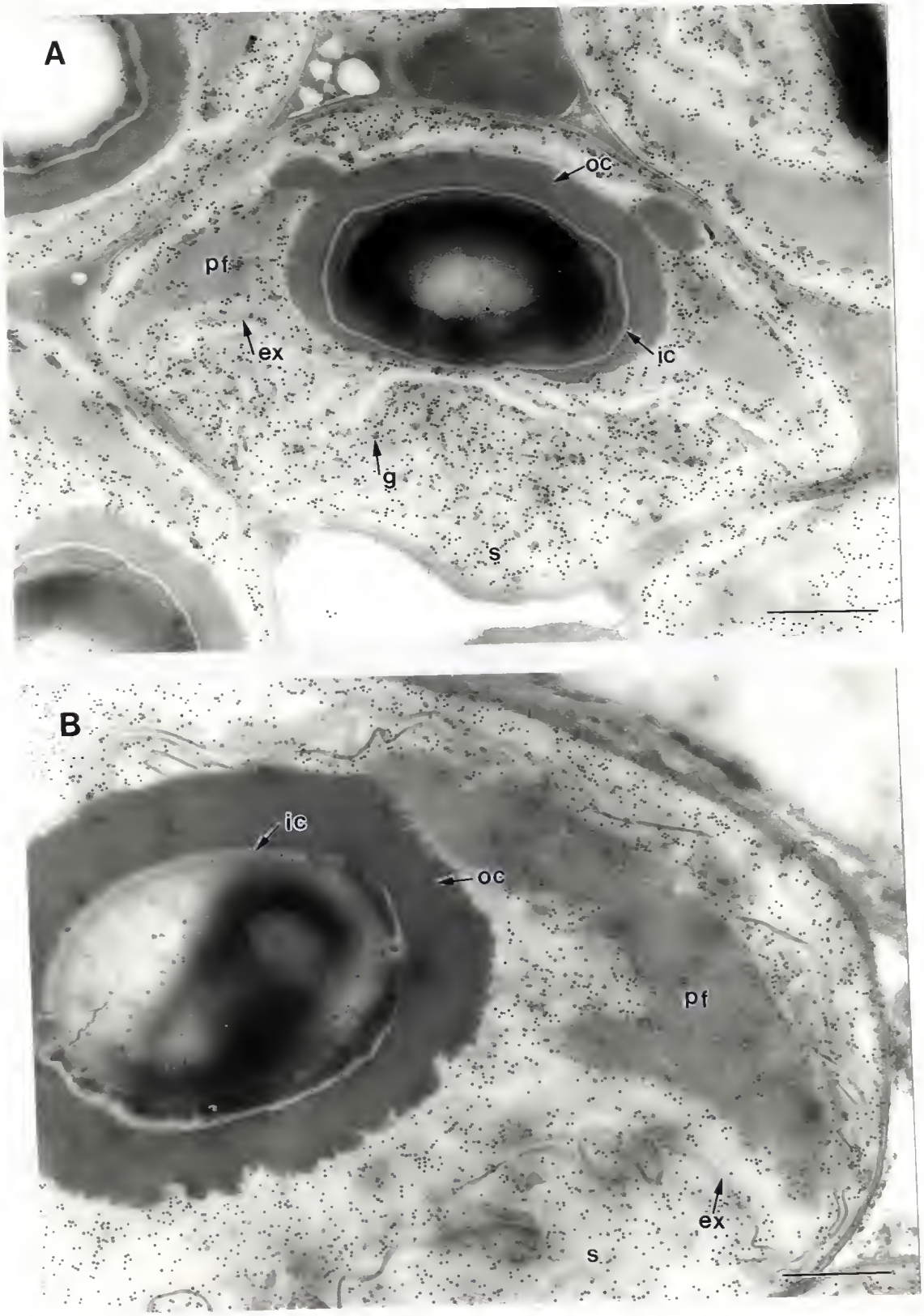
Fig. 3.4. Labeling of endospores of two isolates of *Pasteuria* spp. Scale bars = 0.5 μm. A) LS-1 isolate endospore showing labeling over the sporangium (s), exosporium(ex), and parasporal fibers (pf), whereas the outer spore coat (oc), inner spore coat (ic) were not labeled. B) Endospore of the L-1 isolate labeling of the adhesin-associated epitope over the sporangium (s), exosporium (ex), and parasporal fibers (pf). Gold particles (g).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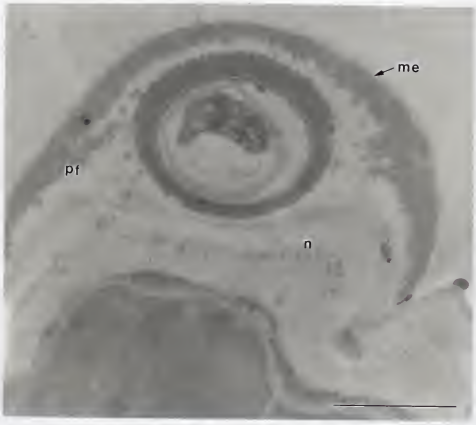

Fig 3.5. Thin section of the *Pasteuria* sp. NA used as a control. Section was treated as the experimental ones, but the first antibody was replaced with non-immune ascites fluid. No gold particles were observed over any structure of the mature endospore (me), parasporal fibers (pf), and nematode cuticle (n). Scale bar = 1 μm.

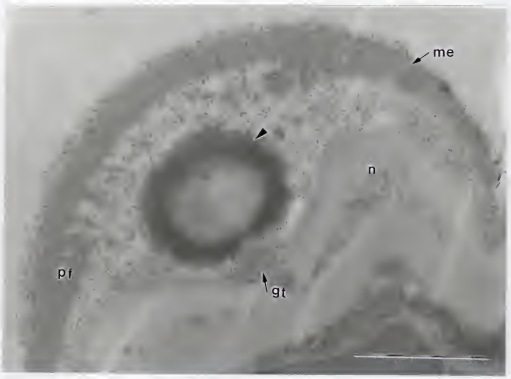

Fig. 3.6. Thin section of a mature endospore (me) of the *Pasteuria* sp. NA. Parasporal fibers (pf) were heavily labeled whereas the germ tube (gt), and the central body (arrow head) were not labeled. Note that the nematode cuticle (n) was not labeled. Scale bar = 1μm.

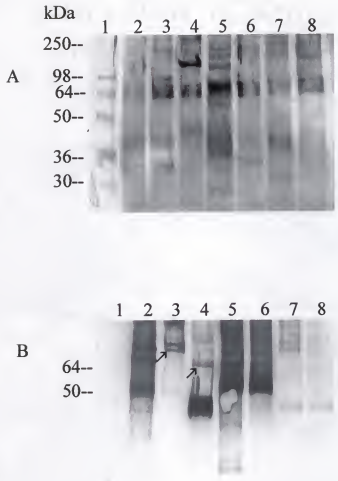

Fig. 3.7. Detection of an adhesin-associated epitope in different strains of *Pasteuria*. Endospore proteins, 600 ng in 20 µl of the appropriate extract plus sample buffer was loaded into each lane. A) Detection of blotted proteins with AuroDye. B) Western blot of blotted proteins probed with anti-P-20 IgM MAb at 1:2,000 dilution in PBST, pH 7.2. Lane 1 - Molecular weight markers, See Blue pre-stained proteins (10 µl/well); Lane 2 - *Pasteuria* sp. NA; Lane 3 - *Pasteuria* sp. S-1; Lane, 4 - C-1; Lane 5 - P1-UFLA; Lane 6 - P-20 isolate of *P. penetrans*; Lane 7 - Spiral nematode-infecting *Pasteuria*; and Lane 8 - Ring nematode-infecting *Pasteuria*.

2001), as well as other forces that may be involved in the attachment, such as hydrophobic interactions (Afolabi et al., 1995; Davies et al., 1996; Esnard et al., 1997; Spiegel et al., 1996).

Immumoblot analyses showed that all isolates of *Pasteuria* share the same adhesin -associated epitope. This epitope also was recognized previously by anti-P-20 IgM Mab in protein extracts from P-120, and B4 isolates of *Pasteuria penetrans* (Charnecki, 1997; Charnecki et al., 1998). Several antigens bearing the same epoitope were recognized by the MAb; however quantitative and qualitative differences were observed among all species and isolates. *Pasteuria* sp. NA, a parasite of *Heterodera glycines*, and P1-UFLA and P-20 isolates from root-knot nematodes have similar profiles of protein bands to those found for *P. penetrans* isolates, which are clearly different from those of protein profiles of extracts from *Pasteuia* sp. S-1, spiral nematode-infecting *Pasteuria*, and ring nematode-infecting *Pasteuria*. *Pasteuria* sp. S-1 and C-1 isolate both showed a certain degree of uniqueness in their protein profiles, which may differentiate them from the other isolates and species. Similar results were obtained by Davies et al. working with polyclonals (1992) and Charnecki (1997) and Charnecki et al, (1998). Davies et al. (1992) found that proteins extracted from three isolates of *P. penetrans*: PP1, PNG, and PCal, probed with a polyclonal antibody previously raised against whole spores of *P. penetrans* PP1, showed qualitative and quantitative differences in the protein profiles, but most of the proteins were conserved. Differences detected with the anti-P-20 IgM MAb confirm not only previous studies with P-120, P-100 and P-20 isolates by Charnecki

(1997) and Charnecki et al., (1998) but also the potential use of this MAb as a probe to detect different species of *Pasteuria*.

The detection of the adhesin epitope over thin sections of endospores of the different species and isolates of *Pasteuria* indicates that this epitope does not confer host specificity, but it may confer virulence. Since this epitope has been proven to be involved in the attachment of *P. penetrans* P-20 endospores to its nematode host, *M. arenaria* race 1, its broad distribution on thin sections of endospores of other species and isolates of *Pasteuria* with different host specificity indicates that it is only one component of the attachment process. The results of these studies show that the adhesin associated-epitope, which was not found associated with any of several species of *Bacillus* previously examined (J. Harrison and J. F. Preston, unpubl.; Schmidt et al., 2001) is unique for strains and species of *Pasteuria*.

CHAPTER 4
SYNTHESIS OF SMALL, ACID-SOLUBLE SPORE PROTEINS IN *Pasteuria penetrans*

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr is a Gram-positive mycelial-and endospore-forming bacterium known from around the world. It has shown great potential as a biological control agent of root-knot nematodes (*Meloidogyne* spp.) (Chen and Dickson, 1998; Dickson et al., 1994; Trudgill et al., 2000). For instance the application of 10,000 endospores per gram of soil effectively suppressed the peanut root-knot nematodes on peanut (Chen et al., 1997b).

Phylogenetic studies using 16S rDNA of *Pasteuria ramosa* (Ebert et al., 1996), *P. penetrans* (Anderson et al., 1999) and *Heterodera glycines*-infecting *Pasteuria* (Atibalentja et al., 2000) have placed *Pasteuria* spp. in a clade with some species of *Bacillus*. *Bacillus* spp. have had many aspects of their life cycle, such as growth factors, sporulation genes, and proteins, well characterized, therefore they can provide an appropriate model to study species of *Pasteuria*.

Spore of various species of *Bacillus* have a number of small, acid-soluble spore proteins (SASPs), which are synthesized during the first 3-4 hours sporulation (Setlow et al., 1992). SASPs comprise approximately 5 to 10% of the protein in dormant spores of *Bacillus* and *Clostridium* species (Cabrera-Martinez and Setlow, 1991; Setlow, 1988).

The major function of these proteins is to bind to and protect the DNA from environmental trauma. The main types of SASPs found in *B. subtilis* are termed the α/β type (Connors et al., 1986) and γ type (Hackett and Setlow, 1984). Previous studies indicated that α/β type-SASPs are DNA-binding proteins, and their binding to the DNA protect it from UV irradiation (Manson and Setlow, 1986; Setlow and Setlow, 1987). Studies in vivo and in vitro have shown that α/β-type SASP are non-specific DNA-binding proteins, which trigger a conformational change in DNA from a B-like to an A-like structure (Mohr et al., 1991; Setlow et al., 1992). The α, β and γ from make up about 18, 18, and 36% of the total SASPs of *B. subtilis* strain 168 with molecular weights of 5,900, 5,900, and 11,000 kDa (Johnson and Tipper, 1981). Another funtion of these proteins is to be a source amino-acids for new protein synthesis during spore germination and outgrowth (Setlow, 1981). Setlow and Primus (1975) showed that spores lack several important pools of amino acids and germinating spores lack several key amino acid synthetic enzymes.

Amino acid sequences of α/β type-SASP are highly conserved within and across species of a variety of endospore-forming bacteria (Cabrera-Martinez, 1991; Setlow, 1988). Setlow (1988) analyzed the SASPs of 19 species of *Bacillus* and found that 27 residues were conserved exactly and 11 were similar. He also showed that this conservation exists between the tested *Bacillus* species and various species of *Clostridium*. Therefore the SASPs may be an useful model to study SASPs in *P. penetrans*. The objective of this study was to select an antibody against a synthetic

peptide (sequence: Cys-Ser-Val-Gly-Gly-Glu-Ile-Thr-Lys-Arg-Leu-Val), a conserved sequence of amino acid in *Bacillus* SASPs, and use it as a probe to detect SASPs in *P. penetrans*.

Materials and Methods

Pasteuria penetrans Endospores Source

The source and the procedure to obtain the endospore of *P. penetrans*, P-20 strain, used in this study was the same as for Chapter 2.

Bacillus subtilis Spore Source

Purified and lyophilized spore of *B. subtilis*, ATCC strain 6051 was kindly provided by Dr. James F. Preston and Josh Loomis, Microbiology and Cell Science Department, University of Florida, Gainesville, Florida.

Extraction and Determination of SASPs from *P. penetrans* and *B. subtilis*

The extraction of SASPs from spores of *B. subtilis* and *P. penetrans* was performed according to Johnson and Tipper (1981). Clean and dry spores of *B. subtilis* (10 mg/ml) and endospore suspension of *P. penetrans* (1×10^8/µl) in 2N HCl were used to perform the acid rupture of spores and extraction of SASPs (APPENDIX A). Protein estimation was performed by a micro-protein assay based on the Bradford method (Bradford, 1976), according to the manufacturer's instructions (BioRad, Hercules, CA). Standard curves were generated using bovine serum albumin (BSA) (Sigma, St. Louis, MO), and colorimetric measurement was performed at 595 nm (Hewlett Packard 8451A Diode Array spectrophotometer, Palo Alto, CA).

Conjugation of SASP Peptide to Carrier Proteins

A synthetic peptide (sequence: Cys-Ser-Val-Gly-Gly-Glu-Ile-Thr-Lys-Arg-Leu-Val), which was designed based on a conserved region of *Bacillus* SASPs, was synthesized by the Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL. Two milligrams of the peptide were added to each of two 1.5 ml microfuge tubes containing 300 µl of conjugation buffer (83 mM Sodium phosphate buffer, pH 7.2, 0.9 M NaCl, 0.1 M EDTA, 0.02% azide) according to the manufacturer's instructions (44895 sulfoLink® Kit, Pierce Chemical Company, Rockford, IL). Twenty microliters of the peptide mixture was added to a 1.5 ml microfuge tube and stored at 4 °C until use. In separate 1.5 ml microfuge tubes, pre-activated keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) were mixed with conjugation buffer, pH 7.2 at a concentration of 2 mg per 200 µl. The peptide mixture (280 µl) was combined with each protein mixture (200 µl) in 1.5 ml microfuge tube and incubated at room temperature for 2 hours prior to purification.

Purification of the Conjugates

Each of the conjugate mixtures was added to a gel filtration column, which was washed previously three times with the purification buffer (0.083 M sodium phosphate buffer, pH 7.2, 0.9 M NaCl) according to the manufacturer's instructions (44895 sulfoLink® Kit, Pierce Chemical Company, Rockford, IL). Using the purification buffer as diluent, 15 fractions of 1 ml each were collected and proteins were determined by measuring the absorbance at 280 nm, to verify which of the fractions had the most conjugate (Hewlett Packard 8451A Diode Array spectrophotometer, Palo Alto, CA). The

peak fraction for each conjugate was retained and concentrated using a Centricon ™ 3 device according to the manufacturer s' instructions (Amicon Inc., Beverly, MA). The final concentration was estimated for each of the conjugate based on Abs_{280} nm. Fractions were stored at 4 °C overnight and used to inject in hens for polyclonal antibody production.

Immunization of Hens for Production of Polyclonal Antibodies

The polyconal antibodies were raised in White Leghorn hens (134-4 and 135-1) against KLH-peptide and (135-2 and 135-3) against BSA-peptide. Approximately 100 µl of KLH-peptide was injected in the wing (subcutaneous), and 100 µl in the footpad of each hen. Two hens were used for each immunogen. Hens were boosted 14 days after the initial injection as follows: approximately 75 µl of KLH-peptide was injected in the wing, and 75 µl in the footpad of each two hens (134-4 and 135-1) , whereas 50 µl of BSA-peptide was injected in the wing and 60 µl in the footpad of each two hens ((135-2 and 135-3). Eggs were collected every day and stored at 4 °C. To monitor the formation of the antibody, two consecutively-laid eggs were combined, extracted, and analyzed for titers against the KLH-peptide and BSA-peptide. Intact egg yolks (~ 15 ml yolk per egg) (APPENDIX B) were removed and rinsed three times with 0.1 M sodium phosphate buffer, pH 7.6. Every two yolks (30 ml/two eggs) were combined and lysed in 120 ml (four volumes) of 0.1 M sodium phosphate buffer, pH 7.6, and stirred (slowly) at room temperature for 30 minutes. Yolk extracts were stored at 4 °C overnight. Activities of the antibodies in each of the egg yolk extracts were determined by ELISA.

Determination of IgY Activities in Yolk Extracts

A general procedure for ELISA was done as described in Chapter 2. Each antigen, KLH-peptide and BSA-peptide diluted at 10,000 in coating buffer (15.00 mM Na_2C0_3, 33.40 mM $NaHC0_3$, 0.0.2% NaN_3) was added to the appropriate wells of the microtiter plate in 75 µl aliquots. Plates containing the antigens were incubated at 4 °C overnight. Extracts from every two egg yolks were screened for antibody activity at 100- and 1,000-dilutions in 10 mM PBST, pH 7.6 (10 mM sodium phosphate buffer, pH 7.6 , 0.9% NaCl, 0.2% Tween 20). After transferring 75 µl of the egg yolk extract to the appropriate wells, plates were incubated at room temperature for 1.5 hours, and washed with PBST four times. One milliliter of each yolk suspension was centrifuged at 10,000 × g for 2 minutes prior to dilutions. The secondary antibody, anti-chicken IgG conjugated to alkaline phosphatase (Sigma) at 1:2,000 in PBST was added to each well (75 µl per well), and incubated for another 1.5 hours at room temperature. The plates were washed with PBST four times again as before. To each well, 75 µl of alkaline phosphatase substrate, 0.1% *p*-nitrophenol phosphate (w/v) (Sigma) in alkaline phosphatase substrate buffer (0.05 M Na_2C0_3, 0.05 M $NaHC0_3$, 0.0005 mM $MgCl_2$) was added. The reaction was monitored by reading the absorbance at 405 nm with an automated microplate reader (Model 2550 EIA, BioRad, Hercules, CA).

Extraction of IgY from Egg Yolk Extracts

Egg fractions that showed high IgY antibody activities were combined in group of two, and the IgY antibodies were purified using polyethylene glycol (Sigma) following

the method described by Polson et al. (1985) (APPENDIX B). The activities of IgY antibodies were determined by ELISA as follows.

Determination of Activities of Purified IgY

IgY fractions extracted from each pool were centrifuged as described before, and each supernatant was diluted to 100, 1,000, and 10,000 in PBST, pH 7.6, and applied to the appropriate well (100 µl/well). The antigens, KLH-peptide, and BSA-peptide were diluted to 10,000 in coating buffer (15.00 mM Na_2C0_3, 33.40 mM $NaHC0_3$, and 0.0 2% NaN_3), and applied to the wells (100 µl/well). The activities of IgY antibodies in the purified fractions were determined by monitoring the absorbance at 405nm as above.

Concentration of Purified IgY using Centripep

The pool (pool 2) that exhibited the highest activity (as shown by ELISA) was concentrated using a Centripep 10 device, following the manufacturers' instructions (Amicon Inc., Beverly, MA). The volume of the anti-peptide IgY obtained after the concentration was 4.5 ml. The affinity of the anti-peptide IgY for SASPs of *B. subtilis* and *P. penetrans* was evaluated by ELISA.

Affinity of Anti-Peptide IgY for SASPs

ELISA procedure was the same as described above. The SASP of *B. subtilis* and *P. penetrans* were diluted to 100 and 1,000 whereas KLH-peptide and BSA-peptide, used as positive controls, were diluted to 10,000 and 100,000 in coating buffer. Anti-peptide IgY (pool 2) was diluted to 100, 1000, and 10,000 in PBST.

Results

Purification of the Conjugates

Fractions number 3 of each purified conjugate had the highest activity. Fraction 3 containing the KLH-peptide or BSA-peptide was used to inject into the appropriate hen. A final concentration of 0.8 mg/ml was estimated for each of the conjugates that was used for immunization.

Determination of IgY Activities in Yolk Extracts

Two White Leghorn hens (134-5 and 135-1) injected with KLH-peptide yielded levels of anti-KLH-peptide antibody (Figs. 4.1-4.4), whereas hens (135-2 and 135-3) injected with BSA-peptide produced very low levels of anti-BSA-peptide antibody (data not shown). These results indicate that KLH-peptide was a better immunogen than BSA-peptide. The highest antibody activities were observed after the antigenic boost of the hens. The egg yolk extracts diluted at 100 showed higher antibody activities than at a dilution of 1,000 dilution regardless of the antigen used in the ELISA, and the hens used to raise the antibodies. The highest level of anti-peptide activity was observed in yolk from eggs laid by hen 134-5 at 16 to 26 days after injection (Fig 4.1), using KLH-peptide as antigen in ELISA. However this high level of activity might be a background due the presence of KLH, in the antigen used. Using BSA-peptide as antigen, hen 134-5, at 16 to 26 days after injection produced some level of activity (Fig. 4.3). This indicated that the activity observed using BSA-peptide as antigen was not due to the antigen's background. Therefore, egg yolk extracts laid by hen 134-5 between 20 and 28 days after injection were used for IgY extraction (Fig. 4.3).

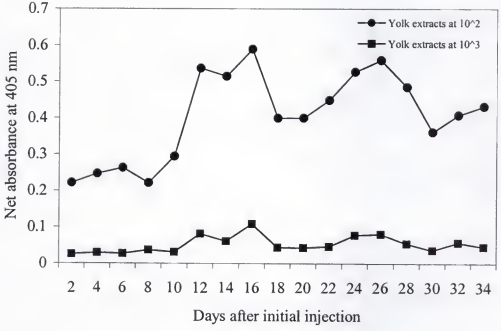

Fig. 4.1. Activities of antibodies in egg yolk extracts collected from hen 134-5, 34 days after injection of 100 µl KLH-peptide as immunogen (80 µg per 100 µl) into the wing and 100 µl into the foot pad. A boost injection was performed at 14 days, 75 µl was injected into the wing and 75 µl into the footpad. Egg yolk extracts were used at 100 and 1,000 dilution in PBST, whereas the antigen (KLH-peptide) was diluted at 10,000 in coating buffer. Absorbance (405 nm) represent readings recorded at 45 minutes after the addition of the substrate in ELISA.

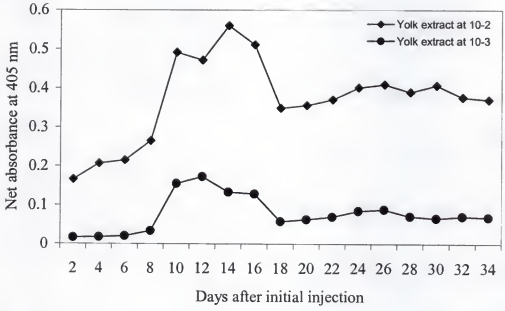

Fig.4.2. Activities of antibodies in egg yolk extracts collected from hen 135-1, 34 days after injection of 100 µl KLH-peptide as immunogen (80 µg per 100 µl) into the wing and 100 µl into the foot pad. A boost injection was performed as in Fig. 4.1. Egg yolk extracts were used at 100 and 1,000 dilution in PBST, whereas the antigen (KLH-peptide) was diluted at 10,000 in coating buffer. Absorbance (405 nm) represent readings recorded at 45 minutes after the addition of the substrate in ELISA.

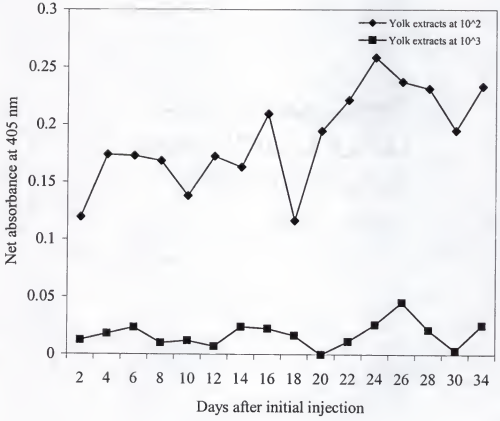

Fig.4.3. Activities of antibodies in egg yolk extracts collected from hen 134-5, 34 days after injection of 100 µl KLH-peptide as immunogen (80 µg per 100 µl) into the wing and 100 µl into the foot pad. A boost injection was performed as in Fig. 4.1. Egg yolk extracts were used at 100 and 1,000 dilution in PBST, whereas the antigen (BSA-peptide) was diluted at 10,000 in coating buffer. Absorbance (405 nm) represent readings recorded at 45 minutes after the addition of the substrate in ELISA.

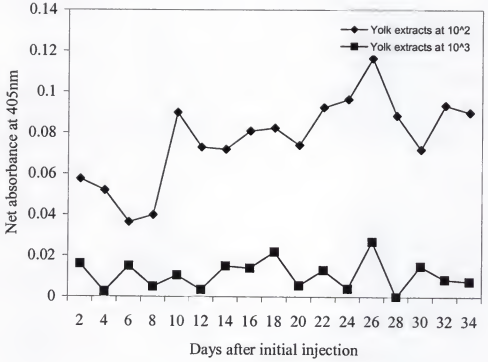

Fig. 4.4. Activities of antibodies in egg yolk extracts collected from hen 135-1 34 days after injection of 100 µl KLH-peptide as immunogen (80 µg/100 µl) into the wing and 100 µl into the pad. A boost injection was performed as in Fig. 4.1. Egg yolk extracts were used at 100 and 1,000 dilution in PBST, whereas the antigen (BSA-peptide) was diluted at 10,000 in coating buffer. Absorbance (405 nm) represent readings recorded at 45 minutes after the addition of the substrate in ELISA.

Extraction IgY from Egg Yolk Extracts

The five pools of egg yolk extracts eggs laid between 20 and 28 days after injection) from hen 134-5 showing high levels of IgY activities were combined by every two polls just before the extraction of IgY antibodies was performed. Each new poll yielded a total of 20, 18, and 16 ml of purified anti-KLH-peptide IgY obtained from a total of 10 eggs.

Determination of Activities of Purified IgY

Antibodies purified from pool 2 showed the highest activity regardless of the planting antigens (Fig. 4.5). The titer of anti-KLH-peptide IgY purified from pool 2 was determined using 100; 1,000; and 10,000 dilutions in PBST, and KLH-peptide and BSA-peptide antigens at 10,000; 100,000; 1,000,000 dilution in coating buffer (Fig. 4.6).

Affinity of Anti-Peptide IgY for SASPs

Anti-peptide KLH IgY isolated from pool 2 recognized SASPs of *B. subtilis* as well as SASP of *P. penetrans* (Figs. 4.7; 4.8), respectively.

Discussion

Hens successfully raised antibodies against the synthetic peptide (sequence: Cys-Ser-Val-Gly-Gly-Glu-Ile-Thr-Lys-Arg-Leu-Val), a conserved sequence of amino acid in SASPs of *Bacillus* spp. KLH was more efficient as a carrier protein than BSA. The anti-peptide KLH IgY purified from yolks extracts laid by hen 134-4 between days 20 to 28 was reactive with SASPs of *B. subtilis* and cross reactive with SASPs of *P. penetrans*. These results indicate that ELISA can be used as a method to detect SASPs of *B. subtilis*

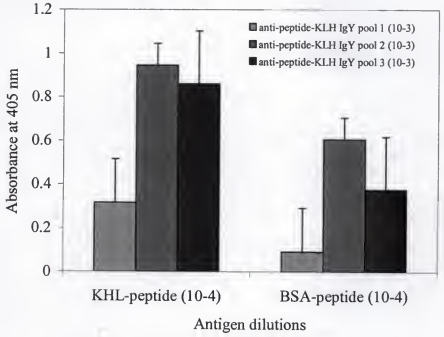

Fig. 4.5. Activities of anti-KLH-peptide IgY antibodies extracted from egg yolk extracts laid by hen 134-5 at 20 to 28 days after injection with KLH-peptide. Antibodies were used at 1,000 dilution in PBST. KLH-peptide and BSA-peptide at 10,000 dilution in coating buffer were used as antigens. Readings were recorded at 45 minutes after the addition of alkaline phosphatese substrate, *p*-nitrophenyl phosphate in ELISA. Lines above bars indicate SE for the average of replicates per assay at $P = 0.05$.

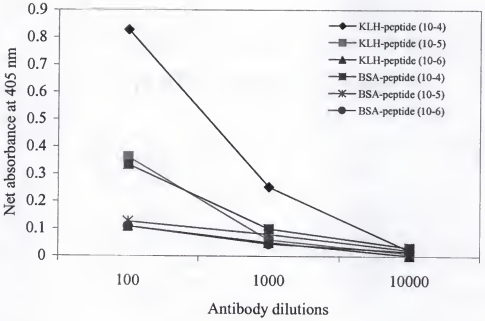

Fig. 4.6. Activities of purified IgY antibodies (pool 2) from egg yolk extracts laid by the hen 134-5. Antibodies were dilute to 100; 1,000; and 10,000 in PBST, pH 7.6 whereas the antigens, KLH- peptide and BSA-peptide, were dilute to 10,000; 100,000; and 1,000,000 in coating buffer. Readings were recorded at 15 minutes after the addition of alkaline phosphate phosphatese substrate, *p*-nitrophenyl phosphate.

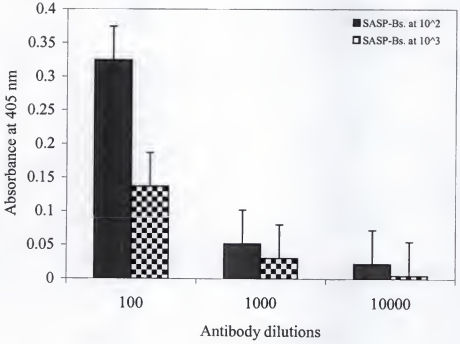

Fig. 4.7. Activities of anti-KLH-peptide IgY antibodies extracted from egg yolk extracts (pool 2) laid by hen 134-5. Antibody was used at 100; 1,000 and 10,000 dilution in PBST. SASP-*Bacillus subtilis* at 100 and 1,000 dilution in coating buffer were used as an antigen. Readings were recorded at 45 minutes after the addition of alkaline phosphatese substrate, *p*-nitrophenyl phosphate in ELISA. Lines above bars indicate SE for the average of replicates per assay at $P = 0.05$.

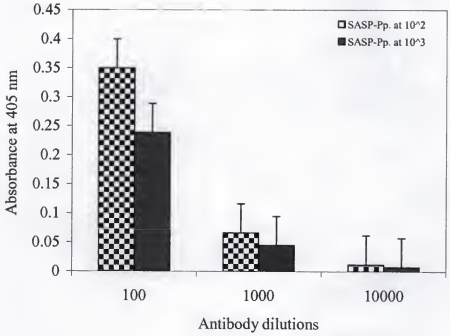

Fig.4.8. Activities of anti-KLH-peptide IgY antibody extracted from egg yolk extracts (pool 2) laid by hen 134-5. Antibody was used at 100; 1,000; and 10,000 dilution in PBST. SASP-*Pasteuria penetrans* at 100 and 1,000 dilution in coating buffer were used as an antigen. Reading were recorded at 45 minutes after the addition of alkaline phosphatase substrate, *p*-nitrophenyl phosphate in ELISA. Lines above bars indicate SE for the average of replicates per assay at $P = 0.05$.

and *P. penetrans*. It also indicated that there was some level of similarity between the SASPs of these two species of endospore-forming bacteria. The anti-peptide KLH IgY will be purified further, and used as a probe to detect SASPs of *B. subtilis* and *P. penetrans*. Further studies are needed to characterize the SASPs of *P. penetrans*, and to compare them to those of *Bacillus* spp. and *Clostridium* spp.

Studies have shown that endospores of *P. penetrans* are resistant to high temperature and desiccation (Stirling, 1984), some nematicides (Freitas, 1997), and microwave oven treatment (Weibelzahl-Fulton, 1996). However, the factors that trigger resistance of endospores to those conditions are yet to be studied. Since SASPs have been implicated. as a source of amino acid during spore germination in *B. subtilis*, they may serve a similar role in *Pasteuria*. Thus they may be key nutrients that trigger germination and allow subsequent vegetative growth. It also will be desirable to determine sequences of the genes encoding SASPs of *Pasteuria* to compare isolates and species using specific probes.

CHAPTER 5
SUMMARY

Plant-parasitic nematodes reduce crop yields by approximately $8 billion a year to producers in the United States and nearly $78 billion worldwide (Society of Nematologists. Committee on National Needs and Priorities in Nematology, 1994). Plant-parasitic nematodes are controlled mainly by crop rotation and chemical nematicides. Rational schemes are oftentimes very difficult to achieve and environmental concerns plus high cost of using nematicides limit their application. The impending ban on methyl bromide has forced the loss of one of the most effective chemical nematicides in use for the past decade.

Pasteuria penetrans (Thorne) Sayre & Starr is one of the most promising biological agents to replace methyl bromide for the control *Meloidogyne* spp. (Chen and Dickson, 1998; Dickson et al., 1994; Trudgill et al., 2000), and has the potential to be used as part of a management program to control root-knot nematodes (Freitas, 1997; Stapleton and Heald, 1991). This bacterium has been reported in root-knot nematode-suppressive soils (Dickson et al., 1994), and also has suppressed root-knot nematodes in greenhouse tests (Brown and Smart, 1985; De Leij et al., 1992; Stirling 1984) as well as in field microplots (Brown et al., 1985, Chen et al., 1997c; Dube and Smart, 1987; Oostendorp et al., 1991; Stirling, 1984; Tzortzakakis and Gowen, 1994; Trudgill et al.,

2000). *Pasteuria*-infected nematodes have significantly reduced egg production capability (Bird, 1986; Bird and Brisbane, 1988).

Pasteuria penetrans completes its life cycle within the pseudocoelom of its nematode host, and unknown signals trigger its germination, growth, and sporulation. The stages of development of *P. penetrans* include: i) recognition and attachment; ii) germination/infection/; iii) vegetative growth; iv) sporulation; and v) release. To be used successfully as a biological control agent, endospores of *P. penetrans* must recognize and bind to molecules on the surface of the nematode-host cuticle. Protein extracts from *P. penetrans* spores have been shown to react with wheat-germ agglutinin, indicating the presence of a carbohydrate ligand on the surface of the spores. These results have provided the basis for a model in which glycoproteins bearing β-1-4-linked N-acetyl glucosamine residues on the surface of the spores, designated as spores adhesins, are recognized by lectins on the cuticle of the nematode (Bird et al., 1989; Charnecki, 1997; Charnecki et al., 1998; Davis and Danks, 1993). Bird et al. (1989) and Charnecki (1997) showed that wheat-germ agglutinin also inhibited endospore attachment to the nematode host.

Previous studies using anti-P-20 IgM monoclonal antibody (MAb), which was selected directly against whole endospores of a *P. penetrans* strain from *Meloidogyne arenaria* race 1 (designated as P-20) demonstrated that this monoclonal antibody blocked the attachment of P-20 to *M. arenaria* race 1 (Charnecki, 1997; Charnecki et al., 1998). The MAb also recognized an epitope that was shared among several polypeptides. In my research, the anti-P-20 IgM MAb was used to characterize an

adhesin-related epitope, as well as to determine whether other species and strains of *Pasteuria* share the same epitope.

The anti-P-20 MAb was used to follow the appearance of an adhesin-associated protein during the development of *P. penetrans* within the pseudocoelom of *Meloidogyne arenaria* (Neal) Chitwood race 1. Tomato, cv. Rutgers, that were inoculated with either second-stage juveniles alone or with endospore-attached second stage juveniles of *M. arenaria* race 1. Nematodes, uninfected or infected, were harvested at 12, 16, 24, and 38 days after inoculation (DAI), and were examined to determine the developmental stage of the bacterium at each window of development. Vegetative growth of *P. penetrans* was observed only in infected nematodes harvested at 12 and 16 DAI, whereas cells at different stages of sporulation and mature endospores were observed at 24 and 38 DAI. Levels of the adhesin-associated proteins were detected by ELISA using the anti-P-20 IgM MAb as the primary antibody. Proteins extracted from uninfected and *Pasteuria*-infected nematodes were resolved by SDS-PAGE, electroblotted, and visualized by Auro Dye or anti-P-20 IgM MAb. Only proteins extracted from infected nematodes harvested at 24 and 38 DAI were recognized by the MAb. ELISA and immunoblot revealed that the amount of the adhesin-associated epitope increased as *P. penetrans* proceeded through sporogenesis. These results indicate that the synthesis of adhesin-related proteins occurred at a certain developmental stage relative to the sporulation process, and are present after mature spores are formed.

Immunocytochemistry techniques were used to investigate where and when the adhesin-associated proteins were formed. Labeling was first observed in stage III of

sporogenesis. Gold particles were clearly observed over the parasporal fibers. Labeling was observed over other structures such as sporangium and exosporium during the sporogenesis process, but labeling was not observed on the cortex, epicortex, inner-spore coat, outer-spore coat, and protoplasm. Immunogold labeling showed that the adhesin-related epitope, which is recognized by anti P-20 IgM MAb on P-20 is equally distributed over endospores of other species and isolates of *Pasteuria*, including P1-UFLA, North American *Pasteuria*, S-1, C-1, LS-1, L-1, and *Rhabditis*-infecting *Pasteuria*. The distribution of the adhesins over several structures of the endospores of the different isolates and species of *Pasteuria* indicates that this epitope does not confer host preference or specificity. Since the epitope has been implicated in recognition and attachment (Charnecki et al., 1998) its broad distribution on *Pasteuria* spp. with different host specificity indicates it is only one component of the attachment process. Other forces associated with surface properties (Afolabi et al., 1995; Kamra and Dhawan, 1998; Mohan et al., 2001) of both endospore and the nematode cuticle may contribute to a cooperative process resulting in recognition, initial interaction, and finally irreversible attachment.

Quantitative and qualitative differences were observed in the protein bands from extracts of *Pasteuria* sp. NA, *Pasteuria* sp. S-1, as well as C-1, P1-UFLA, ring nematode and spiral nematode isolates of *Pasteuria*, compared to P-20 used as control. These results indicate that the epitope that was recognized by the MAb in the P-20 isolate is shared among other isolates and species of *Pasteuria*. This epitope, which was not found associated with any of several *Bacillus* spp. examined previously, is considered to be a

component of a recognition system shared by different isolates and species of *Pasteuria*. (J. Harrison and J. F. Preston, unpubl.; Schmidt et al., 2001) Indirect immunofluorescence was used to visualize the distribution of the adhesin-associated epitope on the surface of whole mature endospores. It revealed that the antigen bearing the epitope does not occur uniformly on the surface of mature endospores of P-20 strain of *P. penetrans*.

This study is the first to report a temporal synthesis of an adhesin-associated epitope during the sporogenesis process of *P. penetrans* and that this epitope is also synthesized by other isolates and species of *Pasteuria*.

APPENDIX A
EXTRACTION OF SMALL, ACID SOLUBLE SPORE PROTEINS FROM SPORES

(Johnson and Tipper, 1981)

1. Add 10 mg of dry and clean spores of *B. subtilis* (10 mg/ml) or 10 μl of an endospore suspension of *P. penetrans* ($1\times10^8/\mu$l) to a 1.5 ml siliconized microtube containing 1 ml 2N HCl.

2. Vortex the suspension until it is homogenous.

3. Incubate at 20 °C for 30 minutes.

4. Centrifuge the suspension at 10,000 × *g* for 10 minutes.

5. Collect the supernatant (S1) in a microtube, and suspend the pellet in 1 ml 2N HCl.

6. Vortex the pellet until it is homogenous.

7. Incubate at 20 °C for 30 minutes.

8. Centrifuge as above.

9. Collect the supernatant (S2) in a microtube.

10. Combine the supernatants (S1 plus S2) and bring it to pH = 5 (use 10N NaOH).

11. Storage at -20 °C.

APPENDIX B
ISOLATION OF IgY ANTIBODY FROM CHICKEN EGG YOLKS

(Modified from Polson et al., 1985)

1. Separate the egg white from the yolk, keeping the yolk.

2. Carefully rinse the egg yolk with 0.1 M NaPO4, pH 7.6, and then place it in a vessel suitable for stirring.

3. Add 4 volumes of 0.1M PO_4 buffer, pH 7.6 and stir for 30 minutes.

4. At this point the extract can be screened for antibody content.

5. The antibody of interest can be further isolated by using the procedure by Polson et al., 1985.

6. Add polyethylene glycol (PEG) (P-2139, Sigma, St. Louis, MO) at 0.035g/ml extract while stirring at room temperature until the PEG is dissolved.

7. Centrifuge at 5000g for 20 minutes at 20 °C.

8. Remove the middle phase, taking care to disrupt the liquid phase as little as possible, and record the volume.

9. Add PEG at 0.085g/ml extract with slow stirring until dissolved, then let stand for 60 minutes.

10. Centrifuge as above for 25 minutes.

11. Decant the supernatant and resuspend the pellet in 0.1M PO_4 buffer, pH 7.6, recording the volume.

12. Slowly add PEG at 0.12 g/ml extract with slow stirring. About 10 minutes after addition of the last of the PEG, centrifuge as per step10.

13. Dissolve the pellet in 0.1M PO_4 buffer, pH 7.6, cooled at 4 °C, then add an equal volume of 50% ethanol (Fisher Scientific, Suwanee, GA), precooled at -20 °C.

14. Stir on ice for 15 minutes, then centrifuge at 10,000 x g for 25 minutes at 5 °C.

15. Dissolve the pellet in 0.1M PO_4 buffer, pH 7.6, and dialyze (10000 MWCO tubing, Pierce Company, Rockford, IL) against 2 to 4 litters of the 0.1M PO_4 buffer, pH 7.6 in cold room overnight.

16. Record the volume after dialysis, and add 1% Na N_3 for preserving.

17. The extract may be stored indefinitely at 4 °C. Do not freeze it.

18. Lastly, a precipitate may form during storage. As long as the precipitate is not disturbed, there is apparently no effect on the titer of the preparation.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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